THE UNIVERSITY OF ALBERTA

CHARACTERIZATION OF ACTIONS OF DOPAMINE IN THE PITUITARY OF THE GOLDFISH, *Carassius auratus*.

BY

ROBERT JOHN OMELJANIUK

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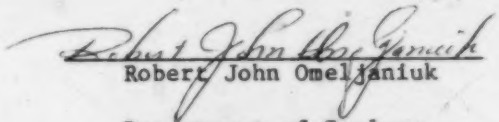

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Characterization of actions of dopamine in the pituitary of the goldfish, Carassius auratus submitted by Robert John Omeljaniuk in partial fulfilments of the requirements for the degree of DOCTOR OF PHILOSOPHY.

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ABSTRACT

The dopamine receptor in the goldfish (Carassius auratus) pituitary and its involvement with inhibition of gonadotropin (GtH) and α-melanocyte stimulating hormone (α-MSH) release was studied.

Dopamine antagonists were injected i.p. into goldfish; sulpiride caused a weak, but significant stereoselective increase in serum concentrations of GtH; domperidone was far more potent than sulpiride. Domperidone, in a dose-related manner, increased serum concentrations of GtH; domperidone action and pituitary uptake of domperidone were temporally correlated. A gonadotropin releasing hormone-analogue (sGnRH-A) increased serum concentrations of GtH in a dose-related manner; sGnRH-A and domperidone acted synergistically to increase GtH release.

In vitro dopamine, in a dose-related manner, inhibited spontaneous GtH and α-MSH release from superfused fragments of pars distalis (PD) and neurointermediate lobe (NIL), respectively; dopamine also inhibited sGnRH-A stimulation of GtH release. Thyrotropin releasing-hormone (TRH), in a dose-related manner, stimulated α-MSH release from NIL fragments; dopamine inhibited TRH action. The stereoisomers of apomorphine were equivalent in inhibiting GtH and α-MSH release from fragments treated with releasing factors. Domperidone, in a dose-related manner, antagonized dopamine action.

[^{3}H]-Spiperone was used to radiolabel the goldfish pituitary dopamine receptor in vitro. The binding of [^{3}H]-spiperone had the characteristics of a receptor: tissue specificity, dependence on tissue quantity, reversibility, saturability, displaceability, specificity of binding with various drugs and a correlation of binding with biological

effects were demonstrated. This is a low-affinity, high-capacity receptor which does not show binding stereoselectivity for apomorphine; domperidone binds avidly to this receptor. The NIL contains significantly greater numbers of this receptor compared to the PD.

The maximum magnitude of the serum GtH response to domperidone varied seasonally; the largest response occurred during advanced stages of seasonal gonadal development. Multiple i.p. injections of sGnRH-A into goldfish caused large chronic increases in serum concentration of GtH; sGnRH-A increased the maximum serum GtH response to domperidone by 4 fold, accompanied by a significant increase in the number of PD dopamine receptors.

In summary, the goldfish pituitary dopamine/neuroleptic receptor is a low-affinity, high-capacity binding site with differential regional distribution in the pituitary. Increased GtH release responses to dopamine ligands *in vivo* were positively related to PD receptor numbers. We propose that this receptor mediates dopamine inhibition of GtH and α-MSH release from the goldfish pituitary gland.

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I. GENERAL INTRODUCTION

Gonadotropin (GtH) release from the pituitary of many teleost fishes is largely regulated by gonadotropin releasing-hormone (GnRH) and dopamine acting as neurohormones; noradrenalin has a direct but minor stimulatory effect on GtH release in the goldfish (for review see Peter, Chang, Nahorniak et al., 1986). Teleosts, in contrast to most other vertebrates, lack a functional hypothalamo-hypophyseal blood portal system; neurosecretory fibres originating from the hypothalamus directly innervate various cell types in the pars distalis (PD) and neurointermediate lobe (NIL) (Ball, 1981). In goldfish, gonadotrophs in the PD are directly innervated by neurons containing GnRH (Kah, Breton, Dulka et al., 1986a) and by neurons containing dopamine (Kah, Dubourg, Onteniente et al., 1986b); melanotroph cells in the NIL are also directly innervated by dopamine neurons (Kah, et al., 1986b) (for a review of vertebrate neurohormonal systems see Peter, 1986). In goldfish, the distribution of GnRH cell bodies and neural pathways in the brain have been investigated using immunocytochemical techniques; GnRH cell bodies in the lateral preoptic region are associated with GnRH neural pathways that run through the ventral hypothalamus and the pituitary stalk to terminate in the proximal PD (Kah, Chambolle, Dubourg & Dubois, 1984a; Kah, Breton, Dulka et al., 1986b). The GnRH contents of various brain regions and the pituitary in female goldfish, at various sexual stages, have been described by immunochemical methods (Yu, Nahorniak, Peter et al., 1987). GnRH acts directly on the gonadotrophs to stimulate GtH release, and Habibi, Van der Loo, Marchant & Peter (1987a) suggest that GnRH action is mediated by a high-affinity GnRH receptor.

Peter & Paulencu (1980) suggested the existence of a GtH release-inhibitory factor (GRIF) in the goldfish brain based on their data from brain lesioning experiments. Immunocytochemical techniques have demonstrated dopaminergic cells in the antero-ventral preoptic area with projections that course through the ventral hypothalamus to the pituitary of the goldfish (Kah, Chambolle, Thibault & Geffard, 1984b). Studies by Chang indicated that of the neurotransmitters tested only dopamine possessed GRIF-activity (for review see Peter et al., 1986). In female goldfish, various steps in the catecholamine biosynthetic pathway were selectively blocked by i.p. injection of specific drugs. In general, serum concentrations of GtH were increased in goldfish injected with drugs which inhibited dopamine synthesis or increased dopamine metabolism; drugs which increased dopamine synthesis or decreased dopamine metabolism lowered serum concentrations of GtH (Chang, Cook & Peter, 1983). Injection of dopamine and apomorphine decreased serum concentrations of GtH in normal female goldfish and depressed the elevated circulating levels of GtH in goldfish with electrolytic lesions in the preoptic area (Chang & Peter, 1983a). Dopamine, apomorphine and bromocryptine, injected i.p., also inhibited the GtH-releasing ability of a GnRH analogue; whereas, pimozide and metoclopramide increased serum concentrations of GtH and potentiated the action of a GnRH analogue (Chang, Peter, Nahorniak & Sokolowska, 1984). Non-dopaminergic agents such as phentolamine, an α -adrenergic antagonist, propranolol, a β-adrenergic antagonist, and octopamine, a sympathomimetic were ineffective in altering serum concentrations of GtH or GnRH analogue action. On the basis of these data Chang et al. (1984) suggested that dopamine inhibits GtH release in goldfish by acting through a dopamine

D2-like receptor. Dopamine inhibition of GtH release has now been demonstrated *in vivo* in a wide number of teleost species by i.p. injection of specific dopamine receptor antagonists such as pimozide and domperidone. Pimozide was used by Chang and Peter (1983b) as well as by Peter, Nahorniak, Sokolowska *et al*. (1985) to antagonize the actions of endogenous dopamine and thereby potentiate GnRH action. Since these studies, numerous findings regarding dopamine inhibition of GtH secretion in various other teleost species have appeared; examples include two species of African catfishes (DeLeeuw, Resink, Rooyakkers & Goos, 1985; DeLeeuw, Goos & van Oordt, 1986), coho salmon (Van der Kraak, Donaldson & Chang, 1986), the common carp (Billiard, Alagarswami, Peter & Breton, 1983), and numerous oriental freshwater cyprinids and cobitids (for review see Peter, Lin & Van der Kraak, 1987). Dopamine inhibition of GtH release from the goldfish pituitary *in vitro* has been demonstrated. Fragments of goldfish PD and enzymatically dispersed PD cells released GtH spontaneously *in vitro*, and a GnRH analogue stimulated GtH release; dopamine (500 nM) modulated spontaneous GtH release and abolished the GtH-releasing activity of the GnRH analogue (Chang, MacKenzie, Gould & Peter, 1984b). Fitting with the suggestion that GRIF originates in the antero-ventral preoptic region of the brain in goldfish (Peter & Paulencu, 1980), Kah *et al*. (1984b) demonstrated a dopaminergic nucleus in this location and a dopaminergic pathway to the pituitary by immunocytochemical methods in the goldfish. More recent evidence indicates that electrolytic lesions placed in the anterior preoptic area of the goldfish brain destroyed local catecholaminergic nuclei and resulted in degeneration of neural fibres, immunopositive to tyrosine hydroxylase, that innervated the pars distalis, in particular

the gonadotrophs; similar immunopositive fibres in the neurointermediate lobe appeared unaffected (Kah, Dulka, Dubourg et al., 1987). Accompanying these affects were increased exocytotic profiles observed in the gonadotrophs and large increases in serum concentrations of GtH (Kah et al., 1987).

In contrast to teleosts, the role of dopamine in regulation of luteinizing hormone (LH) release in mammals is still unresolved. Considerable evidence suggests that dopamine does not directly affect LH secretion in the rat (for review see Barraclough, Wise & Selmanoff, 1984) but does favour an indirect stimulatory role for dopamine on LH release via actions at the hypothalamic level (Kalra & Kalra, 1983; Kalra, 1986). Nonetheless, there is evidence which indicates a direct inhibitory role of dopamine on LH release in rabbits (Dailey, Tsou, Tindal & Neill, 1978), rats (Nicoletti, Ambrosi, Giammartino et al., 1986; Shaban & Terranova, 1986), and humans (Leblanc, Lachelin, Abu-Fadil & Yen, 1978).

The role of dopamine in regulation of release of some other mammalian pituitary hormones is clearly defined. For example dopamine, released from the hypothalamus and transported via the hypothalamo-hypophyseal portal system to the pituitary, interacts with dopamine D2 receptors on lactotrophs of the anterior pituitary and melanotrophs of the intermediate lobe to inhibit the release of prolactin (Cronin, 1982) and α-MSH (Cote, Eskay, Frey et al., 1982) respectively. In teleosts, dopamine has also been implicated as an inhibitor of prolactin (for review see Peter & Fryer, 1983) and α-MSH (Olivereau, 1978; Olivereau, Olivereau & Lambert, 1987) release.

Consequently, in light of the importance of dopamine in regulation of GtH secretion and reproduction in goldfish, the purpose of this investigation was to examine the existence and nature of the goldfish pituitary dopamine receptor with respect to regulation of GtH, as well as α-MSH, release. The major findings in this thesis are arranged into 4 main chapters. The first of these chapters deals with in vivo studies of the receptor specificity of dopamine inhibition of GtH release, the second chapter describes in vitro findings of the receptor specificity of dopamine inhibition of GtH and α-MSH release, the third chapter outlines the binding parameters of the goldfish pituitary dopamine/neuroleptic receptor, and the fourth chapter addresses the concept of seasonal changes in dopamine inhibition of GtH release and the integration of GnRH and dopamine action in regulation of GtH release. The GtH values in this study were determined by a radioimmunoassay based on a carp GtH standard, kindly supplied by B. Breton, as described by Peter, Nahorniak, Chang & Crim (1984); α-MSH was measured by a radioimmunoassay based on a synthetic α-MSH standard, kindly supplied by H. Vaudry, as described by Vaudry, Tonon, Delarue et al. (1978).

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II. *In vivo* evaluation of dopamine receptor-mediated inhibition of gonadotropin secretion from the pituitary of the goldfish.

INTRODUCTION

There is evidence that dopamine acts directly as an endogenous inhibitor of gonadotropic hormone (GtH) secretion in a wide variety of teleost fishes (for review see Peter, Chang, Nahorniak *et al*. 1986). Of the endogenous catecholamines, only dopamine appears to inhibit directly the spontaneous and releasing factor stimulated release of GtH *in vivo* (Chang, Cook & Peter,1983; Chang & Peter, 1983a; Chang, Peter, Nahorniak & Sokolowska, 1984b) and *in vitro* (Chang, MacKenzie, Gould & Peter, 1984b) in the goldfish.

In contrast to teleosts, the role of dopamine in regulating luteinizing hormone (LH) secretion in mammals has not been completely resolved. Evidence exists for dopamine inhibition of LH secretion in rabbits (Dailey, Tsou, Tindall & Neill, 1978) and man (Leblanc, Lachelin, Abu-Fadil & Yen, 1976) whereas in the rat the bulk of evidence suggests a lack of direct dopamine influence on LH secretion (for review see Barraclough, Wise & Selmanoff, 1984) or a potentially indirect stimulatory role of dopamine on LH release (for review see Kalra & Kalra, 1983). In many respects the DA-mediated inhibition of GtH secretion in goldfish is reminiscent of dopamine D2 receptor-mediated inhibition of prolactin release in the rat (McDonald, Sibley, Kilpatrick & Caron, 1984); specific dopamine D2 antagonists increase the release of prolactin and GtH in the rat and goldfish, respectively.

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As dopamine appears to play an important role in the regulation of GtH secretion in goldfish, and hence its sexual status, the purpose of this study was to investigate further the receptor specificity of dopamine inhibition of GtH secretion and to define the use of domperidone, a high-affinity specific dopamine D2 receptor antagonist (Baudry, Martres & Schwartz, 1979), to manipulate goldfish pituitary function.

MATERIALS AND METHODS

Experimental animals:

Male and female common or comet variety goldfish (Carassius auratus; approximately 26 g, 10-12.5 cm) were obtained from Grassyforks Fisheries Co., Martinsville, IN, U.S.A. or from Ozark Fisheries, Stoutland, MI, U.S.A. Fish were maintained for several weeks in flow-through aquaria at controlled (± 1 °C) ambient temperatures (7-19 °C, annual range) with simulated natural photoperiod, and were fed twice daily with commercial trout food. Before an experiment, fish were acclimated at 12 °C and a 16 h photoperiod, and fed twice daily for 7-10 days. The day preceding an experiment, fish were anaesthetized in tricaine methanesulphonate (0.5 g/l), weighed and identified by an opercular tag (No. 1005 Size 1 monel; National Band and Tag Co., Newport, KY, U.S.A.). In all cases, animals were anaesthetized before any handling. At the end of all experiments, fish were anaesthetized and killed by spinal transection just posterior to the medulla oblongata.

Drug treatments:

A. Stereospecificity of serum GtH response to dopamine antagonists (September, 1985).

Goldfish were given a single i.p. injection of vehicle (control fish), (+)- or (-)-sulpiride (Ravizza s.p.a., Milan Italy), or domperidone (Janssen Pharmaceutica, Beerse, Belgium). Dopamine antagonists were dissolved in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, U.S.A.) then diluted to final injection concentration with

propylene glycol, and injected in a vehicle of DMSO:propylene glycol (1:9, v/v) at a rate of 5 ml/kg body weight using a 21 gauge (g) 37 mm needle on a 250 ul Hamilton glass syringe. Blood samples were collected 24 h after injection by puncture of caudal vessels using a 25g 16 mm needle mounted on a 1.0-ml tuberculin syringe. Collected blood was transferred to 1.5-ml polypropylene centrifuge tubes, kept on ice for several hours, and centrifuged at approximately 13000g X 10 min at 4 °C. Collected serum was transferred to 500 ul polypropylene centrifuge tubes, frozen on dry ice and stored at -20 °C until radioimmunoassay for GtH (Peter, Nahorniak, Chang & Crim, 1984).

B. Effect of various DA antagonists on serum GtH levels in the absence or presence of [D-Arg^{6}, Trp^{7}, Leu^{8}, Pro^{9}-N-ethylamide]-LHRH (sGnRH-A) (October, 1985).

Goldfish were injected i.p. with vehicle (control fish), or sGnRH-A, or DA-antagonists in the absence or presence of sGnRH-A (kindly supplied by J. Rivier and W. Vale, Salk Institute, La Jolla, CA, U.S.A). Dopamine antagonists were dissolved in DMSO; sGnRH-A was dissolved in distilled water and then both were diluted to final concentration for injection (dopamine antagonists at 10 umol/kg body weight; sGnRH-A at 10 ug/kg body weight) independently or together, in a vehicle of DMSO:propylene glycol:distilled water (9:81:10, v/v) in a total volume of 5 ml/kg body weight. Dopamine antagonists that were tested included domperidone, spiperone, pimozide (Janssen Pharmaceutica), cis-flupenthixol (H. Lundbeck & Co., Valby, Denmark), fluphenazine (Schering Corporation, Bloomfield, NJ, U.S.A.), and metoclopramide (Sigma). Blood was collected 24 h after injection and assayed for GtH.

C. Time course of ^{3}H-radioactivity uptake into blood, pituitary, gonad, and brain after i.p. injection of [^{3}H]-domperidone (March, 1986).

Goldfish received a single 20 ul i.p. injection, containing approximately 1.5 uCi [^{3}H]-domperidone (New England Nuclear, (NEN) Boston, MA, U.S.A.; 30.6 Ci/mmol) and 800 nmol unlabelled domperidone in a vehicle of DMSO:propylene glycol (1:2, v/v) (using a 27g 13 mm needle mounted on a 1.0-ml tuberculin syringe in a Hamilton repeating dispenser). Samples of blood were taken from groups of fish at various times after injection. The fish were then killed and the pituitary, whole brain, portions of gonads, and aliquots of whole blood collected and processed for liquid scintillation counting (LSC). All samples were digested with a combination of 1.0 ml Protosol (NEN) and 200 ul distilled water, according to the manufacturer's instructions, in sealed glass scintillation vials at 50 ^{o}C for 3 days. Blood, gonad and brain samples were subsequently decolourized with 200 ul aliquots of a 20 % (wt/v) benzoyl peroxide (BDH Chemicals Canada, Edmonton, AL, Canada) solution in toluene, according to the manufacturer's instructions. All samples were counted in 10 ml of a toluene-based LSC cocktail and correction was made for variation in counting efficiency due to quenching. Separate pools of pituitaries taken at 6 and 24 h after injection, whole blood from each sampling time between 3 and 96 h after injection, and injectable material were extracted in chloroform and methanol (9:1, v/v) and chromatographically evaluated by thin layer chromatography (TLC) on silica gel G TLC-plates (Fisher, Ottawa, Canada) in a solvent system of chloroform:methanol (9:1, v/v). Tissue radioactivity was expressed as radioactivity per g of tissue normalized for a 100 g fish.

D. Time-course of serum GtH-response to domperidone (June, 1985).

Fish received a single injection of vehicle (control fish), domperidone (16.8 umol/kg body weight), [D-Ala^{6}, Pro^{9}-N-ethylamide]-LHRH (LHRH-A; Syndel Laboratories, Vancouver, BC, Canada) (100 ug/kg body weight), or a combination of domperidone and LHRH-A. Domperidone and LHRH-A were prepared for injection as described in Experiment 2. Blood samples were taken from all fish at 3 and 48 h after injection. To reduce potential handling-related stress additional blood samples were taken from one group of fish at 12 and 24 h after injection, while another group was sampled at 6, 18 and 30 h after injection.

E. Effect of dose of domperidone on serum concentrations of GtH (July, 1985).

Domperidone at a given dosage, with or without sGnRH-A (10 ug/kg body weight), was prepared as described in Experiment 2 and injected i.p. Blood samples were collected 24 h after injection.

F. Effect of dose of sGnRH-A on serum concentrations of GtH (July, 1985).

Fish were given a single i.p. injection of vehicle or sGnRH-A at a given dosage, with or without domperidone (5 umol/kg body weight), prepared as described in Experiment 2. Blood samples were collectd 24 h after injection.

Statistics:

Data were analysed by analysis of variance and Duncan's multiple range test. Comparisons were made at the $p=0.05$ level of significance.

RESULTS

A. Stereospecificity of the serum GtH response to dopamine antagonists.

Domperidone at a dosage of 10 umol/kg body weight increased serum concentrations of GtH to over 200 ng/ml (Figure II-1). (-)-Sulpiride caused a significant increase in serum concentrations of GtH compared with vehicle-injected fish at dosages of 10 and 50 umol/kg body weight; (+)-sulpiride between 1 and 50 umol/kg body weight was ineffective in increasing serum concentrations of GtH.

B. Effect of various DA antagonists on serum GtH levels in the absence or presence of [D-Arg^{6}, Trp^{7}, Leu^{8}, Pro^{9}-N-ethylamide]-LHRH (sGnRH-A) (October, 1985).

Of the dopamine antagonists administered without sGnRH-A, only domperidone and pimozide significantly increased serum concentrations of GtH above controls; domperidone was significantly more effective than pimozide in increasing serum concentrations of GtH (Figure II-2). The dopamine antagonists fluphenazine, metoclopramide, pimozide and domperidone given in combination with sGnRH-A caused significantly higher serum concentrations of GtH than sGnRH-A alone, or the respective antagonists alone; antagonist activity at the dose tested, in terms of ability to potentiate the action of sGnRH-A was metoclopramide = fluphenazine < pimozide = domperidone. There were no significant differences in the mean serum concentrations of GtH between fish treated with sGnRH-A alone compared with the fish treated with sGnRH-A plus spiperone and cis-flupenthixol.

C. Time course of ^{3}H-radioactivity uptake into blood, pituitary, gonad, and brain after i.p. injection of [^{3}H]-domperidone (March, 1986).

[^{3}H]-Radioactivity in blood samples increased rapidly following injection of [^{3}H]-domperidone with maximal levels of about 32000 dpm/g occurring 6 h after injection (Figure II-3). Blood radioactivity decreased slowly thereafter to about 4000 dpm/g at 96 h after injection (Figure II-3). Accumulation of radioactivity in whole brain samples was relatively low; maximum brain radioactivity of about 3000 dpm/g occurred at 12 h after injection. In contrast to brain samples, pituitary radioactivity increased rapidly after injection of [^{3}H]-domperidone, to maximum levels exceeding 140,000 dpm/g at 24 h after injection. [^{3}H]-Radioactivity in the brain did not exceed 29 % of blood radioactivity at any sample time (Table II-1). On the other hand, gonadal radioactivity was generally similar to or exceeded blood radioactivity. Of particular interest, pituitary radioactivity was consistently higher than blood or gonad radioactivity at all sample times starting at 12 h after injection. Thin layer chromatography of pituitary extracts taken at 6 and 24 h after injection revealed a single major peak of [^{3}H]-radioactivity indistinguishable from the injectable preparation of [^{3}H]-domperidone. Radioactivity from whole blood samples taken between 3 and 95 h after injection was also indistinguishable from the injectable [^{3}H]-domperidone preparation (data not shown).

D. Time course of serum GtH-response to domperidone (June, 1985).

Serum concentrations of GtH in fish injected with vehicle did not vary significantly over the 48 h following injection (Figure II-4). Domperidone caused a significant increase in serum concentrations of

GtH, compared with those in vehicle-injected fish, at 3, 24, 30, and 48 h after injection. Serum concentrations of GtH in fish injected with LHRH-A were significantly greater than in vehicle injected fish at 3, 12, 18, 24, 30 and 48 h after injection. Injection of domperidone plus LHRH-A caused a significant increase in serum concentrations of GtH at all sample times compared with vehicle-injected controls; the combination of domperidone plus LHRH-A caused higher serum concentrations of GtH than in fish injected with only LHRH-A and at all sample times compared with fish injected only with domperidone.

E. Effect of dose of domperidone on serum concentrations of GtH (July, 1985).

Domperidone given alone caused an increase in serum concentrations of GtH in a dose-related fashion (Figure II-5). The minimum effective dose of domperidone in this experiment was 0.55 umol/kg body weight; the maximum effective dose was 16.8 umol/kg body weight. Domperidone potentiated the action of sGnRH-A in a complex dose-related fashion; the minimum domperidone dosage for potentiation of sGnRH-A action was 0.017 umol/kg body weight. The apparent amount of potentiation of sGnRH-A action by domperidone was constant between 0.017 and 1.68 umol/kg body weight; however, at 16.8 umol/kg body weight, domperidone elicited an even greater potentiation of sGnRH-A action, inducing serum concentrations of GtH of approximately 800 ng/ml.

F. Effect of dose of sGnRH-A on serum concentrations of GtH (July, 1985).

sGnRH-A given alone increased serum concentrations of GtH in a dose-related fashion (Figure II-6). In this experiment the lowest dosage of sGnRH-A effective in causing a significant increase in serum concentrations of GtH was 100 ug/kg body weight. The highest serum concentrations of GtH were approximately 290 ng/ml in response to sGnRH-A (1 mg/kg body weight) in the absence or in the presence of domperidone (5 umoles/kg body weight). Domperidone alone caused a relatively small, but significant, increase in serum concentrations of GtH. Domperidone potentiated the action of sGnRH-A at dosages from 3.3 - 330 ug/kg body weight, but not at a dosage of 1000 ug/kg body weight.

DISCUSSION

Recent findings indicate that dopamine directly modulates the spontaneous release of GtH as well as GnRH-stimulated GtH secretion in goldfish and other teleosts (reviewed by Peter et al., 1986). The purpose of this research was to evaluate the receptor specificity of dopamine inhibition of GtH secretion in goldfish.

Currently, a widely accepted classification for dopamine receptors proposes the existence of two general types, D1 and D2 (Leff & Creese, 1984). The dopamine D1 receptor is insensitive to substituted benzamides whereas the dopamine D2 receptor is very sensitive to substituted benzamides, and can discriminate between the active and inactive stereoisomers (-)- and (+)-sulpiride, respectively (Grigoriadis & Seeman, 1984). It has been demonstrated using mammalian pituitary tissue that the dopamine receptor mediating the inhibition of secretion of prolactin and thyrotrophin (Foord, Peters, Dieguez et al., 1983), and α-melanocyte stimulating hormone (reviewed by Cote, Eskay, Frey et al., 1982) is the dopamine D2 receptor. In this study (+)-sulpiride was ineffective in changing serum concentrations of GtH whereas (-)-sulpiride significantly elevated serum concentrations of GtH, but to a lesser extent than a small dose of domperidone. These results indicate the involvement of a pituitary dopamine D2 receptor in dopamine inhibition of GtH secretion in goldfish.

Chang et al. (1983) suggested that dopamine is involved in inhibiting GtH release in goldfish. Female goldfish were injected with

drugs which blocked selected steps in the catecholamine biosynthetic pathway. Drugs which increased dopamine synthesis or decreased dopamine metabolism lowered serum concentrations of GtH whereas drugs which inhibited dopamine synthesis or promoted dopamine metabolism raised serum concentrations of GtH. Chang et al. (1984b) subsequently demonstrated that i.p. injection of the dopamine agonists apomorphine or bromocryptine inhibited the GtH-releasing ability of an LHRH-analogue whereas the dopamine antagonists pimozide and metoclopramide increased serum concentrations of GtH and potentiated the action of an LHRH-analogue. Phentolamine (an α -adrenergic antagonist) propranol (a β-adrenergic antagonist) and octopamine (a sympathomimetic) had no effect on serum concentrations of GtH or on the action of an LHRH-analogue. Chang et al. (1984b) concluded that the inhibition of GtH secretion in goldfish may be mediated by a dopamine D2-like receptor.

In the present work representative drugs of dopamine antagonist families were examined for their ability to increase serum concentrations of GtH and potentiate the action of sGnRH-A in goldfish; specifically, the drugs investigated were the butyrophenone spiperone, the phenothiazine fluphenazine, the thioxanthene cis-flupenthixol, the substituted benzamide metoclopramide, the diphenylbutylpiperidine pimozide, and domperidone, which belongs to no established family. The present dose of dopamine antagonist (10 umol/kg BW) is similar to the doses of pimozide (0.1 to 10 ug/g BW) previously used (for review see Peter et al., 1986). When administered alone only pimozide and domperidone significantly increased serum concentrations of GtH above those of vehicle-injected fish; serum concentrations of GtH in domperidone-injected fish were significantly greater than those of

pimozide-injected fish. Metoclopramide, fluphenazine, pimozide and domperidone all potentiated the action of sGnRH-A, although pimozide and domperidone were more potent. In preliminary studies, suspensions of pimozide, spiperone, haloperidol or apomorphine were injected (10 ug/g BW) injected alone or in combination with a GnRH analogue into goldfish to compare their effects on serum concentrations of GtH and their effects on GnRH-analogue action. Both pimozide and spiperone elevated serum concentrations of GtH while they and haloperidol potentiated the action of a GnRH-analogue; apomorphine reversed spiperone action (these data are more completely described in Appendix I-A). Interpretation of these data is limited as the drugs tested had different solubilities in the injection vehicle and were not administered on a molar equivalent per body mass dose. An example of the difficulty of comparing the effects of drugs when administered in suspension is illustrated by another study in which goldfish were injected i.p. with pimozide in suspension or domperidone in solution (MW 436 and 542, respectively) at 10 ug/g BW in the absence or presence of a GnRH-analogue. In this study domperidone was 3 to 6 times more potent than pimozide in potentiating GnRH-analogue action (these data are more completely described in Appendix I-B). In contrast, when these ligands were administered in solution at equivalent molar doses, their effects on sGnRH-A action were similar.

The ability of dopamine antagonists to interact with the dopamine D2 receptor has been studied largely in vitro using mammalian brain and pituitary preparations. The potency of these dopamine antagonists is reflected in their 50 % effective doses (ED_{50}) as determined by their ability to displace [^{3}H]-spiperone from incubated striatal tissue

preparations (reviewed by Seeman, 1981). The rank order of potency of these dopamine antagonists as assessed by this *in vitro* technique is: spiperone > domperidone >> fluphenazine > pimozide > cis-flupenthixol >> metoclopramide. Domperidone is the most specific high-affinity dopamine D2 antagonist yet spiperone and fluphenazine are also high affinity specific receptor antagonists; however, at high concentrations spiperone may interact with serotonin receptors. Metoclopramide and pimozide are both relatively selective but low-affinity dopamine antagonists; at high concentrations pimozide may interact with α -adrenergic receptors as well as with calmodulin. Cis-flupenthixol interacts to a greater extent with dopamine D1 rather than dopamine D2 receptors.

The results of this investigation, using a single low dose of dopamine antagonist, provide information on the rank order of activity of these drugs on serum GtH release in goldfish; in combination with sGnRH-A, domperidone = pimozide >> metoclopramide = fluphenazine. Spiperone and cis-flupenthixol at this dosage were ineffective in potentiating the action of sGnRH-A, although in another trial at a higher dosage (approximately four-fold greater) spiperone and haloperidol were more effective than pimozide in potentiating the action of an LHRH analogue (Peter *et al.*, 1986). Previous studies indicate that spiperone injected as a suspension, rather than as a solution, potentiated the action of a GnRH-analogue in the goldfish *in vivo* (Appendix I-A,B). Pimozide has been used in goldfish to investigate factors controlling GtH secretion (Chang & Peter, 1983a, 1983b; Chang *et al.*, 1984b; Sokolowska Peter, Nahorniak & Chang, 1985), and the activity of various GtH-releasing hormone analogues (Peter, Nahorniak, Sokolowska *et al.*, 1985). This study represents the first use of domperidone to

modify pituitary function in a lower vertebrate. Part of the difficulty of comparing drug potencies *in vitro* and *in vivo* is that the fate of administered drugs varies widely. Aside from our results on i.p. injected domperidone, virtually nothing is known regarding the metabolic fate of dopamine antagonists in lower vertebrates. Nonetheless, our data on the distribution of i.p. injected domperidone, and, the action of domperidone indicate that it binds to, and blocks pituitary dopamine D2-like receptors to increase GtH release.

Domperidone does not cross the blood-brain barrier in mammals (for review see Brogden, Carmine, Heel *et al.*, 1982). Nonetheless, [^{3}H]-domperidone has been used *in vitro* to identify the binding parameters of dopamine receptors in the mouse striatum (Martres, Baudry & Schwartz, 1978). Our results with goldfish indicate that [^{3}H]-domperidone enters the circulation rapidly after i.p. injection; maximum blood levels of 32000 dpm/g represent aproximately 1 % of the injected dose per g of tissue. The goldfish brain does not appear to accumulate appreciable amounts of [^{3}H]-radioactivity and since the brain:blood ratio of radioactivity is far below unity, we conclude that domperidone does not pass through the blood-brain barrier in the goldfish. In contrast, the gonad:blood ratio of radioactivity is near or greater than unity; this suggests that gonadal radioactivity is at least in free equilibrium with the blood and that the gonad may even accumulate [^{3}H]-radioactivity, albeit to a limited extent. Of interest was the rapid and dramatic concentration of [^{3}H]-domperidone by the pituitary; maximum levels of pituitary [^{3}H]-domperidone were approximately 4.25 % of the injected dose per g pituitary. These results suggest the existence of a pituitary factor which binds domperidone,

presumably dopamine D2 receptors on various pituitary cells. In a preliminary investigation [^{3}H]-spiperone was injected i.p. into goldfish maintained at 12 ^{o}C and 12 h photoperiod; ^{3}H-radioactivity in the plasma and pituitary increased rapidly after injection of [^{3}H]-spiperone with relatively maximal levels attained at 6 h after injection; pituitary radioactivity was consistently greater than plasma radioactivity (expressed as ^{3}H-radioactivity per gram of tissue) (Appendix I-C).

The initial time course of [^{3}H]-domperidone accumulation in the pituitary correlates well with the time course of serum GtH response to injected domperidone and to domperidone plus LHRH-A. The dosages of unlabelled domperidone in the two experiments were similar (17 vs 20 umol/kg body weight). Administered alone, domperidone caused a transient but significant increase in serum concentration of GtH at 3 h after injection. Blood and pituitary [^{3}H]-radioactivity increased significantly 30 min after injection of [^{3}H]-domperidone, and by 3 h after injection, pituitary radioactivity, per unit weight of tissue, exceeded blood radioactivity by almost twofold. Since it has been shown that the effects of dopamine and metoclopramide on GtH secretion *in vitro* are virtually immediate (Chang *et al.*, 1984a), the present data suggest that a critical dosage of domperidone may have to accumulate in the pituitary *in vivo* before GtH secretion is sufficiently increased to be reflected by a significant increase in circulating levels of GtH. In comparison, the time course of action of a GnRH analogue, or a combination of the GnRH analogue and pimozide injected i.p. into goldfish at 12 ^{o}C (Appendix I-D) is similar to that of sGnRH-A or sGnRH-A + domperidone in fish. Increased temperature (20 ^{o}C) served to accelerate the onset of GnRH-analogue action and potentiation of this

action by pimozide; at 20°C the absolute maximal serum GtH response occurred earlier than at 12°C and was, as well, somewhat diminished (these data are more completely described in Appendix I-D).

This is the first study to evaluate the potency of a dopamine antagonist, in a dose-related fashion, to increase serum concentrations of GtH in a lower vertebrate. Serum concentrations of GtH, at 24 h after injection, increased in response to i.p. injected domperidone alone in a dose-related fashion. The minimum effective dose of domperidone was 0.55 umol/kg body weight with an ED_{50} of approximately 9.4 umol/kg body weight (ED_{25} approximately 5.5 umol/kg body weight). Serum concentrations of GtH at 24 h were generally significantly higher than those at 6 h after i.p. injection with domperidone (Appendix I-E). In comparison, in unconscious male rats, systemically administered domperidone increased plasma prolactin concentrations in a dose-related fashion with an ED_{25} = 0.155 umol/kg body weight, with the maximum effect occurring at or before 30 min after injection (Cocchi, Gil-Ad, Parenti et al., 1980).

It is becoming increasingly evident from our studies in goldfish that gonadotropin-releasing hormones and dopamine antagonists modify the serum GtH response to one another. In the present study, sGnRH-A increased serum concentrations of GtH in a dose-related fashion in fish of both sexes in July. Serum concentrations of GtH increased linearly (r^2=0.991) in response to sGnRH-A between 10 and 1000 ug/kg body weight (inset Figure II-6). A low dose of domperidone (5 umol/kg body weight) increased the sensitivity of the serum GtH response to sGnRH-A, and at higher dosages of sGnRH-A the relationship between sGnRH-A and serum

concentrations of GtH became asymptotic-like, reaching presumed maximal levels of 295 ng/ml. This concentration of GtH was identical to that produced by the same high dose of sGnRH-A (1000 ug/kg body weight) in the absence of domperidone. Peter et al. (1985) demonstrated that in goldfish of mixed sex in April i.p. injection of a low dose of pimozide in suspension (2.2 umol/kg body weight) potentiated the action of sGnRH-A in a dose-related fashion. In contrast, a high dose of pimozide (22 umol/kg body weight) administered to male fish in May potentiated the action of sGnRH-A, but sGnRH-A did not increase serum concentrations of GtH in a dose-related manner in the presence of that dose of pimozide.

In summary, the findings of this study support and extend the concept of pituitary dopamine receptor-mediated inhibition of GtH release in goldfish (reviewed by Peter et al., 1986). We propose that a dopamine D2-like receptor subtype on gonadotrophs in the goldfish pituitary can be stereospecifically blocked by the active optical enantiomer of sulpiride. Domperidone, which has a much higher affinity than (-)-sulpiride for the mammalian dopamine D2 receptor, is more potent than (-)-sulpiride in increasing serum concentrations of GtH in the goldfish. Domperidone injected i.p. is excluded from the brain but is concentrated in the pituitary where small amounts apparently are sufficient to cause significant increases in GtH secretion. The sensitivity and magnitude of this dose-related increase in circulating concentrations of GtH in response to domperidone are increased by sGnRH-A. These data strongly suggest that endogenous dopamine and GnRH act in concert to control GtH secretion. Moreover, dopamine and GnRH modify the GtH release-response to each other.

Table II-1. Variations in tissue ^{3}H-radioactivity relative to blood ^{3}H-radioactivity after intraperitoneal injection of [^{3}H]-domperidone into goldfish*.

Time after injection (h)	Tissue radioactivity relative to blood ^{3}H-radioactivity (%)		
	Brain	Gonad	Pituitary
0.5	7	141	74
1	7	92	93
3	11	37	194
6	8	103	216
12	15	218	453
24	16	118	785
48	28	238	1361
96	26	248	976

*Sample radioactivity was first expressed as:

$$\frac{[\text{radioactivity (dpm)} / \text{tissue mass (g)}] \times \text{fish mass (g)}}{100 \text{ (g)}}$$

to yield the tissue radioactivity per gram normalized for a 100 g fish. This value was then divided by the mean blood radioactivity per gram normalized for a 100 g fish (at the same sampling time), then multiplied by 100 to give a per cent tissue:blood radioactivity ratio.

Figure II-1: Stereospecificity of the serum gonadotropin (GtH) response to dopamine antagonists (September, 1985).

Serum concentrations of GtH (ng/ml) in goldfish sampled 24 h after i.p. injection with (+)-sulpiride (▲), (-)-sulpiride (●), or domperidone (■) at various doses. Values are means +/- S.E.M. (n=10). ★ $p < 0.05$ compared with vehicle-injected fish (Duncan's multiple range test).

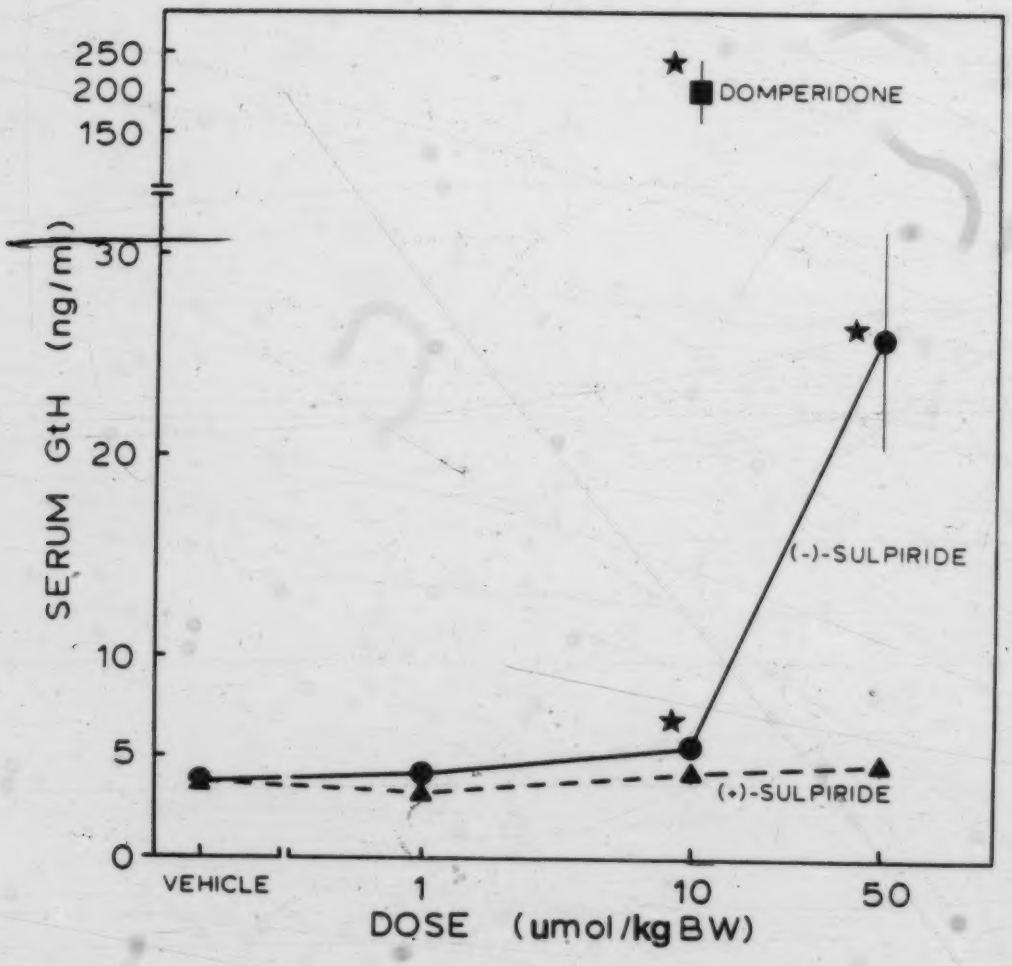

250
200
150
30
20
10
5
0
SERUM GtH (ng/ml)
DOMPERIDONE
(-)-SULPIRIDE
(+)-SULPIRIDE
VEHICLE
1
10
50
DOSE (umol/kg BW)

Figure II-2: Effects of various dopamine antagonists on serum concentrations of gonadotropin (GtH) in the absence or presence of [D-Arg^{6}, Trp^{7}, Leu^{8}, Pro^{9}-N-ethylamide]-LHRH (sGnRH-A) (October, 1985).

Serum concentrations of GtH in goldfish sampled 24 h after i.p. injection with dopamine antagonists (10 umol/kg body weight) in the absence (open bars) or presence (stippled bars) of sGnRH-A (10 ug/kg BW). VEH=vehicle, SPI=spiperone, FLP=cis-flupenthixol, FLF=fluphenazine, MET=metoclopramide, PIM=pimozide, DOM=domperidone. Values are means +/- S.E.M. (n=8). ▼ $p < 0.05$ compared with vehicle -injected fish; △ $p < 0.05$ compared with sGnRH-A-injected fish (Duncan's multiple range test).

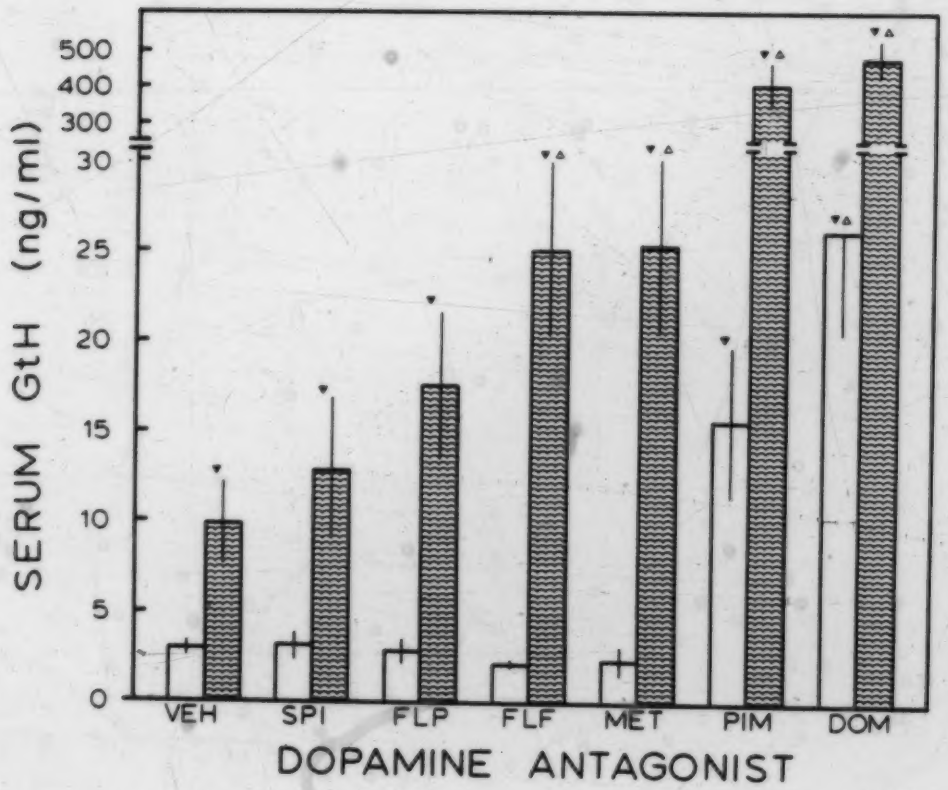

SERUM GtH (ng/ml)
500
400
300
30
25
20
15
10
5
0
VEH
SPI
FLP
FLF
MET
PIM
DOM
DOPAMINE ANTAGONIST

Figure II-3: Time-course of [^{3}H]-radioactivity uptake into blood, pituitary, gonad and brain after i.p. injection of [^{3}H]-domperidone (March, 1986).

[^{3}H]-Radioactivity (X 10^{-3} dpm/g) adjusted for 100 g fish in the blood (■), brain (▲), and pituitary (●) of goldfish after i.p. injection of [^{3}H]-domperidone. Values are means +/- S.E.M. (n=10). Average weight of whole brain and pituitary were 160 mg and 2.579 mg, respectively.

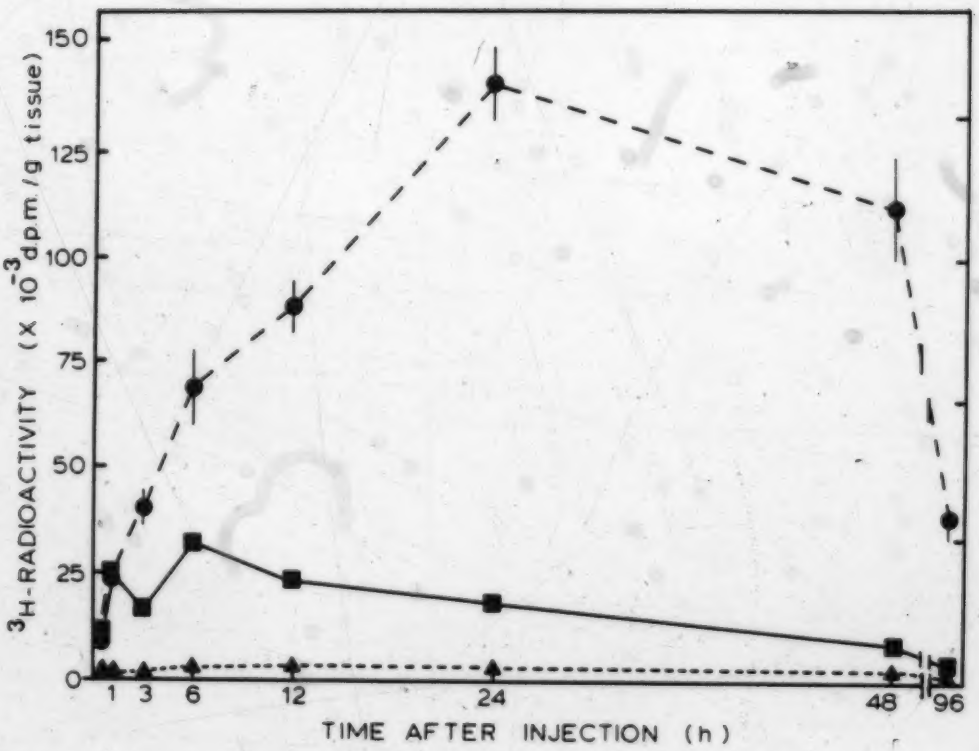

150
125
100
75
50
25
0
^{3}H-RADIOACTIVITY (X 10^{-3} dpm/g tissue)
1
3
6
12
24
48
96
TIME AFTER INJECTION (h)

Figure II-4: Time-course of the serum gonadotropin (GtH) response to domperidone (June, 1985).

Serum concentrations of GtH (ng/ml) following i.p. injection of vehicle (⬢), domperidone (16.8 umol/kg BW, ●), [D-Ala^6, Pro^9-N-ethylamide]-LHRH (LHRH-A) (100 ug/kg BW, ■) or a combination of DOM and LHRH-A (▲) into goldfish. Values are means +/- S.E.M. (n=10). ▼ $p < 0.05$ compared with vehicle-injected fish; △ $p < 0.05$ compared with LHRH-A-injected fish (Duncan's multiple range test).

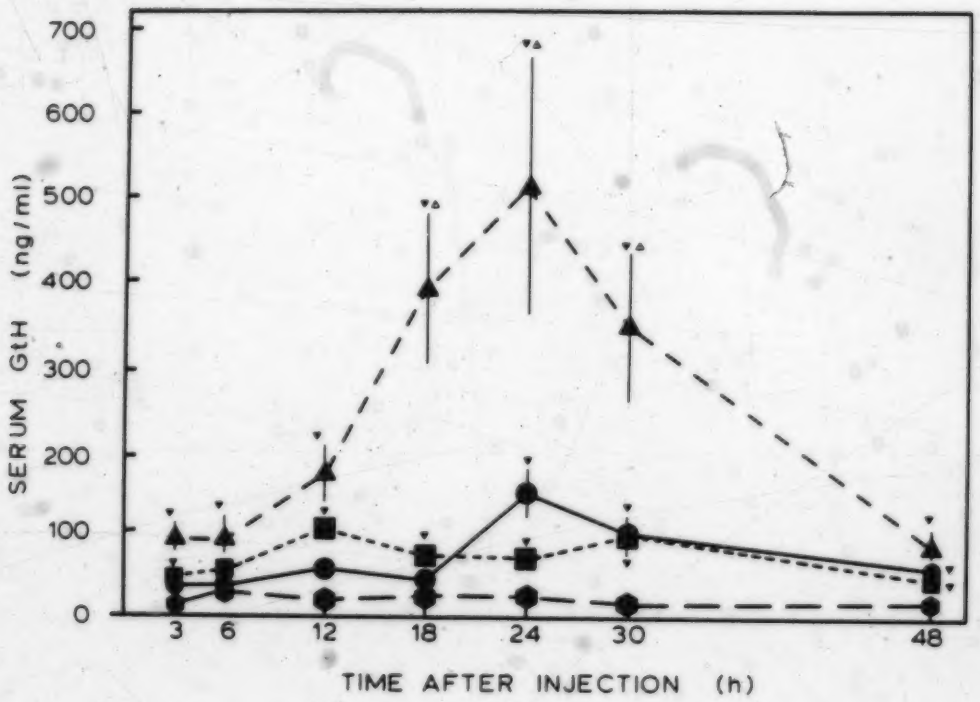

700
600
500
400
300
200
100
0
SERUM GtH (ng/ml)
3
6
12
18
24
30
48
TIME AFTER INJECTION (h)

Figure II-5: Effect of dose of domperidone on serum concentrations of gonadotropin (GtH) (June, 1985).

Serum concentrations of GtH (ng/ml) in goldfish 24 h following intraperitoneal injection of various doses of domperidone in the absence (●) or presence (▲) of [D-Arg^{6}, Trp^{7}, Leu^{8}, Pro^{9}-N-ethylamide]-LHRH (sGnRH-A). Values are means +/- S.E.M. (n=8). ★ $p < 0.05$ compared with vehicle-injected fish; ☆ $p < 0.05$ compared with sGnRH-A-injected fish (Duncan's multiple range test).

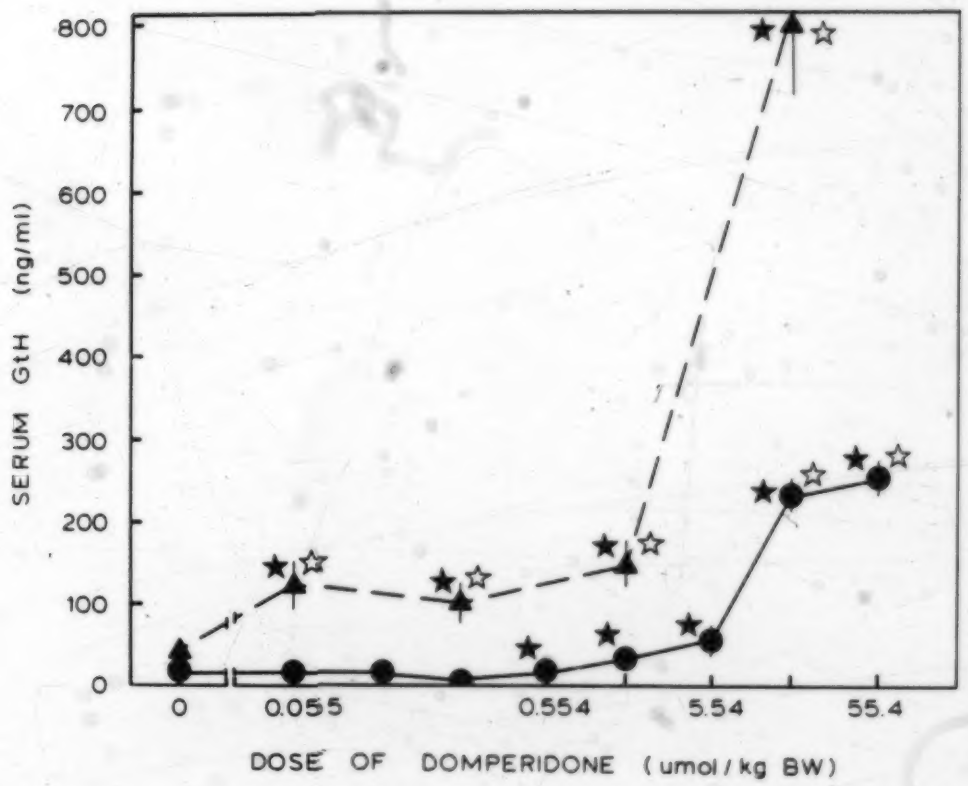
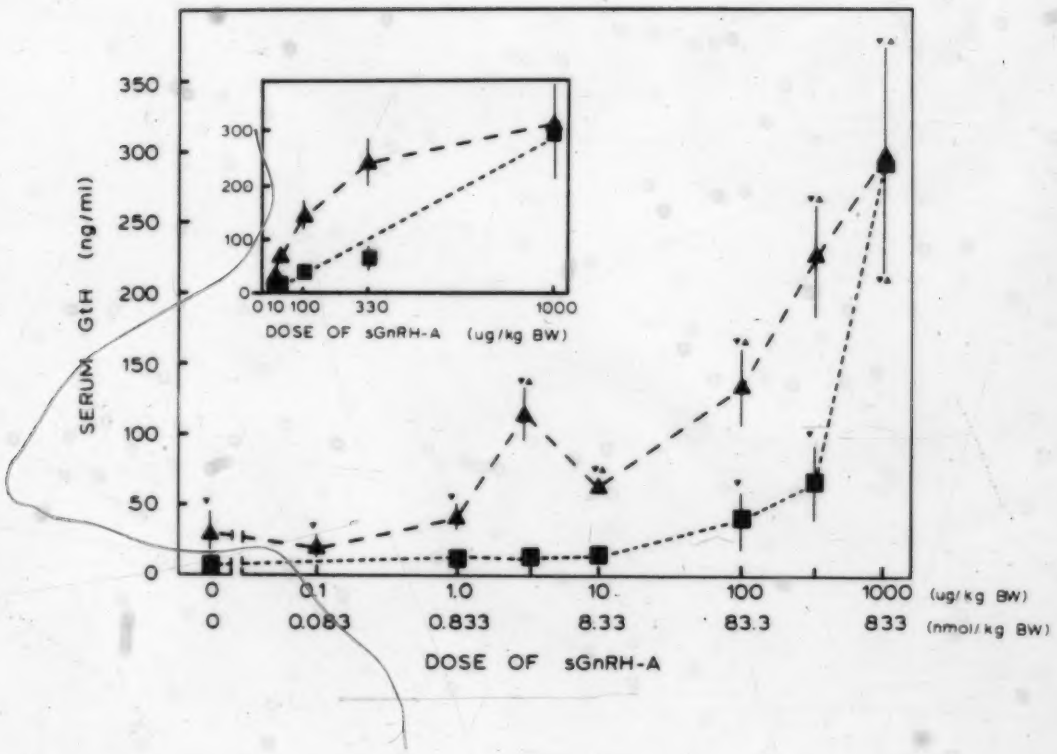

800
700
600
500
400
300
200
100
0
SERUM GtH (ng/ml)
0
0.055
0.554
5.54
55.4
DOSE OF DOMPERIDONE (umol/kg BW)

Figure II-6: Effect of dose of [D-Arg^{6}, Trp^{7}, Leu^{8}, Pro^{9}-N-ethylamide]-LHRH (sGnRH-A) on serum concentrations of gonadotropin (GtH) (July, 1985).

Serum concentrations of GtH (ng/ml) 24 h after i.p. injection of various doses of sGnRH-A in the absence (■) or presence (▲) of domperidone. Values are means +/- S.E.M. (n=9). ▼ $p < 0.05$ compared with vehicle-injected fish; △ $p < 0.05$ compared with sGnRH-A-injected fish (Duncan's multiple range test). Inset: Serum concentrations of GtH (ng/ml) replotted as a linear function of dose of sGnRH-A.


SERUM GtH (ng/ml)
350
300
250
200
150
100
50
0
0 10 100 330 1000
DOSE OF sGnRH-A (ug/kg BW)
0 0.1 1.0 10 100 1000 (ug/kg BW)
0 0.083 0.833 8.33 83.3 833 (nmol/kg BW)
DOSE OF sGnRH-A

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III. *In vitro* actions of dopamine and domperidone, a specific dopamine receptor antagonist, in regulation of gonadotropin (GtH) and α-melanocyte stimulating hormone (α-MSH) release from the pituitary of the goldfish, *Carassius auratus*.

INTRODUCTION

Gonadotropin (GtH) secretion from the pituitary of teleost fishes is regulated predominantly by the stimulatory actions of gonadotropin releasing hormone (GnRH) and the inhibitory actions of dopamine (for review see Peter, Chang, Nahorniak *et al.*, 1986). Also, dopamine has been implicated as an inhibitor of the release of other teleost pituitary hormones, notably prolactin (for review see Peter & Fryer, 1983), and α-melanocyte stimulating hormone (α-MSH) (Olivereau, 1978; Olivereau, Olivereau & Lambert, 1987). The teleost pituitary lacks a functional hypothalamo-hypophyseal portal system, but individual cell types in the pars distalis (PD) and neurointermediate lobe (NIL) are innervated directly by neurosecretory fibres originating from the hypothalamus (Ball, 1981). Gonadotrophs are directly innervated by neurosecretory fibres immunoreactive to GnRH (Kah, Breton, Dulka *et al.*, 1986a) and dopamine (Kah, Dubourg, Onteniente *et al.*, 1986b). Dopamine fibres also terminate directly on α-MSH cells in the goldfish NIL (Kah *et al.*, 1986b).

Dopamine inhibition of α-MSH release is well established in mammals (for review see Tilders, Berkenbosch & Smelik, 1985) and amphibians (Adjeroud, Tonon, Gouteux *et al.*, 1986; for review see Tonon, 1984). Dopamine exerts its action on α-MSH release (Tilders, Berkenbosck & Smelik, 1985) and on prolactin release (Cronin, 1982) through the dopamine D2 receptor subtype. In contrast, the role of dopamine in regulating luteinizing (LH) hormone release in mammals is

controversial. Some data indicate an inhibitoy role of dopamine on LH release *in vivo* in rabbits (Dailey, Tsou, Tindall & Neill, 1978), rats (Nicoletti, Ambrosi, Giammartino *et al.*, 1986; Shaban & Terranova, 1986), and humans (Leblanc, Lachelin, Abu-Fadil & Yen, 1978); however considerable other evidence indicates a lack of direct influence of dopamine on LH secretion in the rat (for review see Barraclough, Wise & Selmanoff, 1984) and favours an indirect stimulatory role for dopamine on LH release via actions at the hypothalamic level (Kalra & Kalra, 1983; Kalra, 1986).

We have previously demonstrated receptor specificity for the direct dopamine inhibition of GtH release in goldfish *in vivo* (this thesis, Chapter II) and demonstrate the existence of a [^{3}H]-spiperone/neuroleptic binding site (dopamine/neuroleptic receptor) in the goldfish PD and NIL (this thesis, Chapter IV). The purpose of this study was to investigate the dopamine receptor-specificity for inhibition of GtH and α -MSH release from the goldfish pituitary *in vitro*.

MATERIALS AND METHODS

Experimental animals:

Male and female goldfish (approximately 40 g, 13-15 cm) of common or comet varieties were obtained from Grassyforks Fisheries Co., Martinsville, IN, U.S.A. or from Ozark Fisheries, Stoutland, MO, U.S.A. Fish were maintained for several weeks in flow-through aquaria at simulated ambient temperature (7-19 ^{o}C) and photoperiod (8-17 h photophase), and fed twice daily with commercial trout food. In all cases animals were anaesthetized (tricaine methanesulphonate, 0.5 g /l) prior to any handling. To harvest pituitary glands, fish were anaesthetized, killed by spinal transection just posterior to the medulla oblongata, and the pituitary removed and placed into ice-cold, sterile buffer (HEPES-buffered Hank's salt solution: Hank's salts; HEPES, 0.02 M; bovine serum albumin, 2 g/l; pH=7.4 at 17^{o}C).

Superfusion of fragments of the PD and NIL:

Pituitaries were transferred to buffer at 17^{o}C, and the PD was surgically separated from the NIL using opposed scalpels while observing under a dissecting microscope. The PD and NIL lobes were pooled, and individual lobes (approximate volume 3 - 4 mm^{3}) dissected into approximately eight equivalent fragments. PD- and NIL fragments were collected into 12 X 75 mm polystyrene culture tubes and rinsed with 4.0-ml aliquots of buffer three times by alternately centrifuging (200g X 3 min) the tubes and discarding the supernatant. Fragments were equally distributed to superfusion chambers and superfused (0.25 ml/min) with buffer at 17^{o}C for 2 h prior to drug testing.

A superfusion chamber consisted of a vertically-oriented, modified 1.0-ml syringe barrel; a filter-paper disc (Whatman No. 1) obstructed the luer-outlet at the bottom of the chamber and supported a 0.150-ml packed bed of G-50 Sephadex, which in turn supported the pituitary fragments. An additional 0.150-ml packed bed of G-50 Sephadex was layered on top of the pituitary fragments. The top of the superfusion chamber was sealed with a rubber plunger, through which a 15 gauge (g), 37 mm stainless steel injection needle had been inserted; the volume of the superfusion chamber was 0.45 ml. A 0.48 m length of LKB-silicone tubing (1.3 mm I.D., #2030-962, LKB-Produkter AB, Bromma, Sweden) was connected to an 18 g, 37 mm syringe-needle which was in turn attached to the luer end of the syringe barrel; buffer was drawn through the chamber at 250 ul/min by a peristaltic pump (#2132 MicroPerpex, LKB). Buffer was supplied into the top of the superfusion chamber via the 15 g, 37 mm needle connected to 0.27 m of Silastic medical grade tubing (1.5 mm I.D., Dow Corning Corp., Midland, MI, U.S.A.) connected to an 18 g 37 mm needle which rested in buffer or test substance. Fractions were collected into 10 X 75 mm glass (PD-eluate) or polystyrene (NIL-eluate) tubes. Tubes were capped, frozen by partial immersion in ethanol cooled by dry ice and stored at -20 ^{o}C for subsequent analysis by RIA.

Hormone determinations:

Samples from superfusion systems containing PD fragments were analysed for GtH content by an RIA based on carp GtH (Peter, Nahorniak, Chang & Crim, 1984). Samples from superfusion systems containing NIL fragments were analysed for α-MSH content using an RIA for α-MSH (Vaudry, Tonon, Delarue et al., 1978), using a highly characterized

antibody (Jegou, Tonon, Leroux et al., 1981) adapted to these in vitro samples.

Development, validation and application of this α-MSH RIA, as well as assessment of the specificity of the primary antibody (#810103), have been described in detail elsewhere (Vaudry et al., 1978); our method involves minor modifications of this protocol.

The protocol for radioiodination of α-MSH was based on a modification of the method of Hunter & Greenwood (1962). In a 5.0-ml polystyrene tube 1 ug of synthetic α-MSH in 10 ul of 4 mM HCl was mixed with 20 ul of acetate buffer (100 mM, pH=4.0) and 20 MBq of ^{125}I in 5 ul (100 mM NaOH). Iodination was initiated by addition of chloramine-T (20 ul of 0.250 g/l in phosphate buffer, 0.5 M, pH=7.6) and stopped after 15 sec by addition of sodium metabisulfite (20 ul of 3 g/l in phosphate buffer, 0.5 M, pH=7.6). Separation of radiolabelled α-MSH from free ^{125}I was performed by adsorption to QuSO G-32 glass powder (Philadelphia Quartz Co., Philadelphia, PA, U.S.A.) as described for adrenocorticotrophic hormone (ACTH) purification (Vaudry, Vague, Dupont et al., 1975). The radioiodinated α-MSH was divided into aliquots, frozen and stored at -20 °C.

Preparation of incubation mixtures for RIA was as described by Usatagui, Oliver, Vaudry et al. (1976). Two days after the start of incubation 200 ul of normal rabbit serum (1:127 v/v final dilution in assay buffer: sodium barbital, 0.02 M, pH=8.6; bovine serum albumin, 3 g/l; mercaptoethanol, 1:500 v:v, Sigma; Aprotinin, 0.010 g/l, Sigma), and 200 ul of anti-rabbit goat gamma-globulins (1:63 v:v final dilution in assay buffer) were added separately to assay tubes and incubated at

4° C for an additional 2 days. RIA tubes were then centrifuged at 1000 g X 30 min at 4° C, the supernatant was decanted and the pelleted precipitate counted by gamma-spectroscopy. Probit (Logit) analysis of the bound radioactivity was used to estimate α-MSH content.

Unlabelled α-MSH standard inhibited the binding of [^{125}I]-α-MSH to anti-α-MSH antibody in a dose-related manner (Figure 1). Probit analysis and parallel line analysis (Pekary, 1979) indicated that this dose-related inhibition was linear (r^2=0.976) between 50 and 6250 pg/ml of α-MSH standard. Eluate from superfused PD- or NIL fragments also inhibited the binding of radiolabelled α-MSH in a linear (r^2=0.968-0.998) dose-related manner with slopes that were identical to that of the α-MSH standard. The principal difference between eluate from PD- and NIL-superfusion systems was that the α-MSH content of NIL samples was approximately 8-fold greater than that of PD-samples; the presence of α-MSH in PD-samples may have been due to the presence of some NIL tissue in the PD-superfusion chamber; we do not exclude the possibility that the PD may actually produce and release small quantities of α-MSH.

Specific investigations:

A. Time-course of spontaneous GtH and α-MSH release from superfused pituitary fragments.

PD- or NIL fragments in 4 independent columns were superfused with buffer for 6 h. Eluate was collected as 10-min (2.5-ml) fractions and later analysed for GtH or α-MSH content.

B. (i) Dopamine inhibition of spontaneous GtH and α-MSH release.

PD- and NIL fragments were superfused for consecutive 1 h periods with buffer or a given concentration of dopamine (10^{-10}, 10^{-8}, 10^{-6}, 10^{-4} M, or 10^{-9}, 10^{-7}, 10^{-6}, 10^{-5} M). Eluate from PD- or NIL fragments was collected as 10-min (2.5-ml) fractions and later analysed for GtH or α-MSH content, respectively.

(ii) Effect of domperidone on spontaneous GtH release.

Replicate columns containing PD fragments were superfused for consecutive 1 h periods with buffer then increasing concentrations of domperidone. Eluate was collected as 10-min (2.5-ml) fractions and later analyzed for GtH content.

C. Time course of the elution of [^{125}I]-[DArg^{6}, Trp^{7}, Leu^{8}, Pro^{9}-N-ethylamide]-LHRH (sGnRH-A), GtH, or α-MSH.

A superfusion system, not containing tissue, received a 2.0-min (0.5-ml) dosage of [^{125}I]-sGnRH-A (approximately 1800 cpm, spec. act. approximately 1300 uCi/ug) (Habibi et al., 1987) (unlabelled sGnRH-A was a gift from J. Rivier and W. Vale, Salk Institute, La Jolla, CA, U.S.A.). Eluate was collected in 1.0-min (0.250-ml) fractions and radioactivity of samples determined by gamma-spectroscopy.

Superfusion systems containing PD- or NIL fragments were superfused with buffer and given a 3.0-min (0.75-ml) dose of 2 X 10^{-8} M sGnRH-A or pGlu-His-Pro-NH_2 (TRH, Sigma, St. Louis, MO, U.S.A.), respectively. Eluate was collected in 5.0-min (1.25-ml) fractions and later analysed for GtH or α-MSH content.

D. TRH stimulation of α-MSH release.

NIL fragments were superfused with buffer and at 60 min intervals received a 3-min (0.75-ml) dose of a given concentration of TRH. Each trial consisted of two 10-min (2.5-ml) fractions collected prior to the dose of TRH or buffer, and 4 fractions including and following the dose of TRH or buffer. Eluate was later analyzed for α-MSH content.

E. Effect of multiple treatments of pituitary fragments with releasing factors on GtH or α-MSH release.

In a series of 6 consecutive 1-h trial periods, PD fragments were superfused with buffer for 20 min prior to receiving a 3.0-min (0.75-ml) dose of [Trp^7, Leu^8]-LHRH (sGnRH), or sGnRH-A; NIL fragments were superfused with buffer and were stimulated with multiple doses of TRH (20 nM ($10^{-7.7}$ M), 3.0-min, 0.75 ml). Eluate was collected prior to, during, and following each dose of releasing factor in 10-min (2.5-ml) fractions, and later analyzed for hormone content.

F. Dopamine inhibition of GtH and α-MSH release from pituitary fragments treated with releasing factors.

During 60-min trial periods, PD- or NIL fragments were superfused with buffer or a given concentration of dopamine. Twenty minutes into each trial period, PD- or NIL fragments were given a 3.0-min dose (20 nM ($10^{-7.7}$ M) of sGnRH, or sGnRH-A, or TRH, respectively, in appropriate vehicles. Eluate was collected throughout the periods in 10-min (2.5-ml) fractions, and later analysed for hormone content.

G. Inhibition by (+)- or (-)-apomorphine of releasing factor-stimulated GtH and α-MSH release.

In 60-min trial periods, PD- or NIL fragments were superfused with buffer or a given concentration of (+)- or (-)-apomorphine (Research Biochemicals Inc., Wayland, MA, U.S.A.). Twenty minutes into each trial period, PD- or NIL fragments were given a 3.0-min (0.75-ml) dose of sGnRH or sGnRH-A, or TRH (20 nM), respectively, in buffer or apomorphine. Eluate was collected throughout the trial periods as 10-min (2.5-ml) fractions and later analysed for hormone content.

H. (i) Domperidone antagonism of dopamine inhibition of GtH and α-MSH release from pituitary fragments treated with releasing factors.

In an initial 60-min trial period, PD fragments were superfused with buffer for 20 min prior to receiving a dose of sGnRH or sGnRH-A (3.0-min; 0.75-ml; 20 nM); NIL fragments were similarly stimulated with TRH (3.0-min: 0.75-ml; 20 nM). In subsequent trial periods pituitary fragments were also treated with releasing factors, but were constantly superfused with dopamine (500 nM, $10^{-6.3}$ M) in the absence or presence of given concentrations of domperidone (Janssen Pharmaceutica, Beerse, Belgium). Throughout the experiments eluate was collected in 10-min (2.5-ml) fractions and later analysed for hormone content.

(ii) Effect of domperidone on sGnRH stimulation of GtH release.

In 2 initial 60-min trial periods, PD fragments were superfused with buffer for 20 min prior to receiving a dose of sGnRH (3.0 min; 0.75 ml). In subsequent trial periods PD fragments were similarly treated with sGnRH but were superfused with increasing concentrations of domperidone. Eluate was collected in 10-min (2.5-ml) fractions and later analyzed for GtH content.

Analysis of data:

This investigation attempted to describe the in vitro influences of dopamine and domperidone on the release of GtH and α-MSH from PD- and NIL fragments, respectively, that were or were not treated with hypothalmic factors. The total hormone contents in 6 X 10-min fractions collected during trial periods of superfusion with buffer or a dopamine ligand (in the presence or absence of a dose of releasing factor) were averaged and the mean (± S.E.M.; n=6 fractions) total hormone content was regarded as the net secretory response by pituitary fragments in that column. The initial treatment in each experiment was superfusion with buffer, with or without a dose of releasing factor; responses during subsequent treatments were normalized to represent a percentage of the initial response in buffer (% initial). Where applicable, the normalized net secretory reponses of replicate columns were averaged and the mean (± S.E.M.; n=number of replicate columns) of these values was plotted.

Data were analyzed using the generalized linear model (GLM) procedure of the Statistical Analysis Systems (Halwig & Council, 1979). Hormone release data were fitted to a model that included the main effects of releasing factor and/or drug treatment time, and variation in pituitary response (variation between columns), as well as two- and three-way interactions of these effects. Predetermined comparisons were made using least square means. Differences were considered significant at the $p<0.05$ level. Where indicated, a computerized four-parameter logistic curve-fitting program (ALLFIT) was used for analysis of curves and determination of ED_{50} values (DeLean, Munson & Rodbard, 1978).

This project also investigated the *in vitro* effects of dopamine and domperidone on releasing factor-stimulated hormone release. During each trial period, the amount (ng) of hormone released (in 4 fractions including and following each dose of releasing factor) in excess of pre-releasing factor levels was regarded as the stimulated hormone release-response (pre-releasing factor levels of hormone release were estimated as the mean total hormone content in the 2 fractions preceding the releasing factor dose). Where applicable, the stimulated hormone release responses for replicate columns were averaged and the mean ($\pm$ S.E.M.; n=number of replicate columns) values were used in paired t-tests to determine significant ($p < 0.05$) differences; these data were not suitable for GLM analysis.

RESULTS

A. Effect of dopamine and domperidone on spontaneous GtH release.

Pars distalis fragments superfused with buffer alone spontaneously released GtH at a relatively constant rate over 6 h (Figure III-2A). Dopamine, in a dose-related manner, inhibited spontaneous GtH release from PD fragments in 4 independent columns; for clarity the effects of dopamine on spontaneous GtH release, from only 2 representative columns, are shown in Figure III-2B. In one column, discontinuation of 10^{-5} M dopamine followed by buffer caused a rebound increase in GtH release to a level similar to the initial value; however, there was no significant change in the GtH release rate following discontinuation of superfusion with 10^{-4} M dopamine (Figure III-2B, left panel). In contrast, domperidone (10^{-4} M) reversed the inhibitory action of dopamine (10^{-3} M) and increased GtH release (Figure III-2B, right panel). Superfusion of PD fragments with domperidone alone, $10^{-5.3}$ M, caused variable increases in GtH release; in comparison, domperidone at $10^{-4.3}$ M caused consistent significant increases in GtH release form superfused PD fragments (Figure III-2C). To quantify the effects of dopamine on spontaneous GtH release the mean GtH content of fractions collected from each column during the first 60 min superfusion with buffer alone was designated as the initial GtH release (100 %) and the rest of the data from the column normalized to this value (Figure III-3). Dopamine at concentrations of 10^{-7} M and greater significantly reduced spontaneous GtH release; ALLFIT analysis of these data indicated that dopamine had an ED_{50} of 38.2 uM ($10^{-4.4}$ M) for inhibition of spontaneous GtH release.

B. Time-course of spontaneous α-MSH release and dopamine inhibition of spontaneous α-MSH release.

Neurointermediate lobe fragments superfused with buffer alone spontaneously released α-MSH at a relatively constant rate over 6 h (Figure III-4A); dopamine, in a dose-related manner, inhibited spontaneous α-MSH release from NIL fragments in 2 independent columns (Figure III-4B,C). In one column discontinuation of 10^{-5} M dopamine followed by buffer caused a rebound increase in α-MSH release (Figure III-4C); however, there was no significant change in the α-MSH release rate following discontinuation of superfusion with 10^{-4} M dopamine (Figure III-4B). The mean α-MSH content of fractions collected from each column during the first 60 min of superfusion with buffer alone was designated as the initial α-MSH release (100 %) and the rest of the data from the column was normalized to this value; Figure III-5). Dopamine at concentrations of 10^{-8} M and greater significantly reduced spontaneous α-MSH release. ALLFIT analysis of these data indicated that the ED_{50} for dopamine inhibition of spontaneous α-MSH release was 61 nM ($10^{-7.2}$ M) (Figure III-5).

C. Time-course of the elution of [^{125}I]-[D-Arg^{6}, Trp^{7}, Leu^{8}, Pro^{9}-N-ethylamide]-LHRH (sGnRH-A), GtH, or α-MSH.

Approximately 5 min after the beginning of a 2.0-min dose (1800 cpm total) of [^{125}I]-sGnRH-A, radioactivity began to appear in 1.0-min fractions. Radioactivity was detected in fractions collected between 5 and 20 min after the beginning of the dose; maximum levels of radioactivity were found in a 1.0-min fraction collected in the tenth

min after the beginning of the dose. The calculated recovery of the administered dose was 100.4 %.

Superfusion of PD fragments with sGnRH-A (20 nM; 3 min) stimulated increased release of GtH whose kinetics were temporally synchronized with those of the elution of [^{125}I]-sGnRH-A. The maximum GtH release was observed in fractions collected 5 to 10 min following the start of the sGnRH-A treatement; GtH release returned to pre-stimulatory levels 20 to 25 min following the beginning of the sGnRH-A treatment.

The time course of the α-MSH release-response to TRH was similar to that observed for sGnRH-A stimulated release of GtH. These results indicate that the action of a single dose of releasing factor is rapid in onset and decay. These results indicate that in this system the stimulated release of these hormones to a single dose of releasing factors should be observed for a minimum of 25 to 30 min following the beginning of treatment with releasing factors.

D. TRH stimulation of α-MSH release.

TRH at concentrations of 1 nM and greater caused acute increases in o -MSH release from superfused NIL fragments; notably, the level of α-MSH consistently returned to pre-TRH levels following each α-MSH release-response (Figure III-6). TRH caused significant dose-related increases in stimulated α-MSH release (calculated as the amount of α-MSH released in excess of pre-TRH levels) in 4 fractions including and following each dose of TRH; pre-TRH levels were estimated as the mean α-MSH content of 2 fractions preceding the TRH dose. The data presented in this way conformed to ALLFIT analysis (Figure III-7) which indicated

an ED_{50} of approximately 6.9 nM ($10^{-8.2}$ M) for TRH stimulation of α-MSH release.

E. Effect of multiple treatments of PD fragments with sGnRH or sGnRH-A, and dopamine inhibition of GtH release from PD fragments treated with sGnRH, or sGnRH-A.

Superfusion of PD fragments in 4 replicate columns with 3-min doses of sGnRH (20 nM), spaced 1 h apart, for 6 h, caused relatively consistent acute increases in GtH release; for clarity, results are shown from only 2 representative columns (Figures III-8A,B). At concentrations of 10^{-7} M and greater, dopamine abolished the acute GtH release-response to sGnRH (Figure III-8A,B). Stimulated GtH release (GtH released in excess of pre-sGnRH levels in 4 fractions including and following the sGnRH dose; pre-sGnRH levels were estimated as the mean GtH content in 2 fractions preceding the sGnRH dose) was analyzed as a function of dopamine concentration; however, the data presented in this way did not conform to ALLFIT analysis and an ED_{50} for this action of dopamine could not be estimated. Nonetheless, dopamine caused a significant dose-related inhibition of total GtH release (Figure III-8A,B) (total GtH was regarded as the mean GtH content of 6 X 10-min fractions collected during superfusion with buffer or dopamine, 2 fractions preceding and 4 following the sGnRH dose); these data were normalized to represent a percentage (% initial) of GtH released during the initial 60 min superfusion with buffer and a dose of sGnRH (initial release, 100 %). ALLFIT analysis of these data indicated an ED_{50} of approximately 32 nM ($10^{-7.5}$ M) for dopamine inhibition of GtH release from PD fragments treated with sGnRH (Figure III-9).

In contrast to the consistent acute GtH release responses to sGnRH (Figure III-8), only the first one or two acute pulses of sGnRH-A (20 nM; 3.0 min) caused obvious acute GtH release (Figure III-10A,B); over the course of the experiment, multiple sGnRH-A doses caused a chronic increase in total GtH release. Dopamine, 10^{-8} to 10^{-4} M, decreased the GtH content of fractions collected from PD fragments given a 3-min dose of sGnRH-A (20 nM; 3-min) (Figures III-10A,B). Discontinuation of dopamine (10^{-5} M) followed by superfusion with buffer resulted in a large increase in GtH release during and following sGnRH-A treatment (Figure III-10B); in contrast, the stimulated GtH release-response to sGnRH-A was not restored by superfusion with buffer following 10^{-4} M dopamine (Figure III-10A). The mean GtH content of fractions collected from PD fragments treated with sGnRH-A and initially superfused with buffer alone was designated as the initial GtH release (100 %) and the data from each column were normalized to represent total GtH content as a percentage of the initial GtH release (Figure III-11); ALLFIT analysis of the data represented this way indicated that the ED_{50} for this inhibitory action of dopamine was 350 nM ($10^{-6.5}$ M).

F. Effect of multiple treatments of NIL fragments with TRH on α-MSH release and, dopamine inhibition of α-MSH release from NIL fragments treated with TRH.

Repeated treatment of NIL fragments with TRH (20 nM; 3 min) caused consistent acute α-MSH release responses in fractions collected during and following each dose of TRH (Figure III-12A,B). This stimulated α-MSH release was attenuated by dopamine at 10^{-8} M (Figure III-12A) and was abolished by concentrations of 10^{-7} M dopamine and greater (Figure

III-12A,B); discontinuation of superfusion with dopamine (10^{-5} M) followed by buffer restored the α-MSH release-response to TRH, whereas prior superfusion with 10^{-4} M dopamine maintained a latent modulation of TRH action. Stimulated α-MSH release by TRH was analyzed as a function of dopamine concentration, but these data were insufficient for ALLFIT analysis. However, dopamine caused a significant dose-related inhibition of total α-MSH release from NIL fragments treated with TRH (Figure III-12A,B); the α-MSH content of 6 X 10-min fractions collected from NIL fragments superfused with buffer alone and treated with TRH was designated as the initial release of α-MSH (100 %) and the rest of the data from the column normalized to this value (Figure III-13). These data were sufficient for ALLFIT analysis which indicated an ED_{50} of 19.6 nM ($10^{-7.7}$ M) for dopamine inhibition of total α-MSH release from NIL fragments treated with TRH.

G. Inhibition by (+)- or (-)-apomorphine of releasing factor-stimulated GtH and o -MSH release.

Both (+)- and (-)-apomorphine reduced GtH release from PD fragments treated with sGnRH (Figure III-14A,B). (+)- and (-)-apomorphine modulated and abolished, respectively, the GtH release-response to sGnRH. Stimulated GtH release was analyzed as a function of apomorphine concentration but these data were insufficient for ALLFIT analysis. (+)- and (-)-apomorphine also reduced total GtH release (Figure III-14A,B); the GtH content of fractions collected during the 60-min trial periods of superfusion with apomorphine were normalized, as previously described, to represent a percentage (% initial) of the initial GtH release-response during superfusion with buffer (initial release; 100

%). ALLFIT analysis of these data for (-)-apomorphine indicated an ED_{50} of approximately 5 X 10^{-8} M (Figure III-15); the data for (+)-apomorphine were not suitable for ALLFIT analysis. (-)-Apomorphine at concentrations of 10^{-8}, 10^{-7}, and 10^{-6} M was significantly more effective than (+)-apomorphine in inhibiting total GtH release.

The stereoisomers of apomorphine also reduced GtH release from PD fragments treated with sGnRH-A (Figure III-16A). The GtH release-response to sGnRH-A (GtH released in excess of pre-sGnRH-A levels) was modulated by both stereoismers of apomorphine. (-)-Apomorphine (10^{-7}, and 10^{-5} M) and (+)-apomorphine (10^{-5} M) significantly inhibited total GtH release; the data from each column were normalized to represent GtH content as a percentage of the initial GtH release. (-)-Apomorphine at 10^{-7} M was significantly more active than (+)-apomorphine at inhibiting total GtH release. Data of the apomorphine inhibition of GtH release from PD fragments treated with sGnRH-A were insufficient for ALLFIT analysis.

(-)-Apomorphine (10^{-11} M and greater) and (+)-apomorphine (10^{-7} and 10^{-5} M) significantly reduced the release of α-MSH from NIL fragments treated with TRH (20 nM; 3 min; Figure III-16B). Only (-)-apomorphine abolished the stimulated α-MSH release-response to TRH (α-MSH released in excess of pre-TRH levels). In contrast, both stereoisomers significantly reduced total α-MSH release; these data were normalized to represent α-MSH content as a percentage of the initial α-MSH release. At 10^{-7} M, (-)-apomorphine was significantly more potent than (+)-apomorphine in reducing total α-MSH release. The dosages of (-)- and (+)-apomorphine tested were insufficient for ALLFIT analysis.

H. Effects of domperidone on GtH release from PD fragments treated with sGnRH or sGnRH-A, in the presence or absence of dopamine.

sGnRH (20 nM, 3-min) in the absence of dopamine consistently caused an acute GtH release-response in PD fragments (Figure III-17A,B); superfusion with dopamine (500 nM, $10^{-6.3}$ M) abolished the stimulated GtH release-response to sGnRH and depressed spontaneous GtH release (Figure III-17A). Domperidone, in a dose-related manner, significantly blocked the inhibitory effects of dopamine and restored the stimulated GtH release-response (GtH released in excess of pre-sGnRH levels). Also, domperidone in a dose-related manner blocked dopamine action and at 50 uM ($10^{-4.3}$ M) significantly increased total GtH release compared with GtH release in the presence of dopamine without domperidone (Figure III-17A); these data were normalized to represent GtH release as a percentage of GtH released during superfusion with dopamine (initial, 100%; Figure III-18) but did not conform to ALLFIT analysis. Total GtH release during treatment with 50 uM ($10^{-4.3}$ M) domperidone was slightly, but significantly, greater than that during initial treatment with buffer (Figure III-17A).

Domperidone (50 uM, $10^{-4.3}$ M) significantly increased sGnRH-stimulated GtH release (Figure III-17B) compared with the initial sGnRH-stimulated GtH release in the absence of domperidone. Also, total GtH release was significantly increased by domperidone at 50 uM ($10^{-4.3}$ M) (Figure III-18).

sGnRH-A (20 nM, 3-min) stimulated GtH release from PD fragments superfused with buffer (Figure III-19); dopamine (500 nM, $10^{-6.3}$ M) abolished the stimulated GtH release-response to sGnRH-A. Domperidone did not restore the acute stimulated GtH release-response to sGnRH-A;

however, domperidone antagonized dopamine action and significantly increased total GtH release (Figure III-19); the data from each column were normalized to represent total GtH content as a percentage of initial GtH release (Figure III-20). Domperidone (5×10^{-8} to 5×10^{-5} M) significantly increased total GtH release (compared with GtH release during superfusion with dopamine) to levels comparable with those observed prior to dopamine superfusion; ALLFIT analysis of these data indicated an ED_{50} of 235 nM ($10^{-6.6}$ M) for domperidone reversal of dopamine action (Figure III-20).

TRH (20 nM; 3-min) stimulated α-MSH release from NIL fragments superfused with buffer alone, while dopamine (500 nM, $10^{-6.3}$ M) inhibited the α-MSH release-response to TRH and reduced total α-MSH release (Figure III-21). Domperidone (5×10^{-7} to 5×10^{-5} M) caused a significant, dose-related blockade of the inhibitory action of dopamine on stimulated α-MSH release (α-MSH release in excess of pre-TRH levels); ALLFIT analysis of these data indicated an ED_{50} of approximately 3.0 uM ($10^{-5.5}$ M) (Figure III-22). Also, total α-MSH release was significantly increased by domperidone at 50 uM ($10^{-4.3}$ M) (these data were insufficient for ALLFIT analysis).

DISCUSSION

In this study PD fragments superfused *in vitro* spontaneously released GtH. These findings confirm and extend those of Chang, MacKenzie, Gould & Peter (1984a) who demonstrated that GtH was spontaneously released from goldfish pituitary fragments or enzymatically dispersed pituitary cells. Carp pituitary cells have also been shown to release GtH spontaneously (Ribiero, Ahne & Lichtenberg, 1983). Our results confirm that dopamine modulates spontaneous GtH release; the estimated ED_{50} for dopamine inhibition of spontaneous total GtH release was 3.8×10^{-5} M. By comparison, estimates of the ED_{50} for dopamine inhibition of total GtH release from PD fragments treated with sGnRH and sGnRH-A were 3.2×10^{-8} M and 3.5×10^{-7} M, respectively. Chang *et al*. (1984) also found that dopamine (50 and 500 nM, $10^{-7.3}$ and $10^{-6.3}$ M, respectively) inhibited spontaneous release from pituitary fragments and dispersed pituitary cells. In this study, discontinuation of submaximal doses of dopamine (10^{-5} M) followed by superfusion with buffer alone caused a rebound increase in GtH release; this rebound was not observed following discontinuation of 10^{-4} M dopamine. Increased spontaneous GtH release was also observed during antagonism of dopamine action by domperidone. Chang *et al*. (1984) also observed rebound increases in GtH release following discontinuation of dopamine superfusion (50 and 500 nM, $10^{-7.3}$ and $10^{-6.3}$ M, respectively) of PD fragments and by superfusion with metoclopramide alone. Collectively, these data strongly suggest that the spontaneous release of GtH from pituitary fragments is inhibited by dopamine and is restored by removal of dopamine; also, removal of dopamine can be effected by displacement from its receptors by domperidone. Domperidone itself, at relatively

high doses, increased GtH release from superfused PD fragments; this may be the result of effective competition with endogenous dopamine for receptors at the termini of dopamine neurons on the gonadotroph (implying the existence of more accessible dopamine receptors on the gonadotroph surface away from the synaptoid contact of the dopamine neuron terminus), or some other unknown action of domperidone on gonadotrophs or on neurosecretory terminals in the pituitary. In this case the term "spontaneous release" may be a misnomer as GnRH- and dopamine-neuron terminals remaining in the fragments may continue to exert some influence on GtH release for a period of time.

Our results confirm previous findings that GnRH's stimulate GtH release from goldfish PD fragments superfused *in vitro* (MacKenzie, Gould, Peter *et al.*, 1984; Peter, Habibi, Marchant & Nahorniak, 1987); dispersed carp pituitary cells also secrete GtH in response to stimulation with an LHRH-analogue (Ribiero *et al.*, 1983). In this study, multiple doses of sGnRH caused consistent acute GtH release responses; in contrast, multiple stimulation of PD fragments with sGnRH-A resulted in a chronic increase in GtH release to 149 % of initial levels, in the absence of consistent acute GtH release responses. The mechanism underlying this phenomenon is undefined. However, *in vivo* studies indicate that multiple injections of sGnRH-A into goldfish cause a large chronic increase in serum concentrations of GtH as well as a significant increase in the numbers of pituitary GnRH-receptors (this thesis, Chapter IV). These *in vivo* findings suggest that multiple sGnRH-A treatments of PD fragments *in vitro* may increase "chronic" GtH release possibly through amplification of GnRH-receptor numbers.

Regarding the regulation of α-MSH release from the teleost pituitary, this study provides the first direct evidence of spontaneous α-MSH release from superfused teleost NIL fragments. Dopamine modulated spontaneous α-MSH release with an ED_{50} of 61 nM ($10^{-7.2}$ M). A minor rebound increase in spontaneous α-MSH release was observed following discontinuation of superfusion with 10^{-5} M dopamine but not following 10^{-4} M dopamine. Olivereau (1978) suggested that dopamine inhibits α-MSH secretion in teleosts; in freshwater- or seawater-acclimated eels, multiple injections of pimozide, a selective dopamine antagonist, induced morphological changes in NIL α-MSH-secreting cells indicative of increased α-MSH release; *in vivo* treatments of goldfish with domperidone and sulpiride have induced similar effects in the NIL (Olivereau *et al.*, 1987).

This study provides the first demonstration of TRH-stimulation of α-MSH release from a teleost pituitary. Stimulated α-MSH release was increased by TRH in a significant dose-related manner; α-MSH release consistently returned to basal levels following each stimulated α-MSH release-response. ALLFIT analysis of these data indicated that TRH stimulated α-MSH release with an ED_{50} of 6.9 nM ($10^{-8.2}$ M). In comparison, TRH has a similar potency to stimulate the release of α-MSH from the frog NIL *in vitro* (ED_{50} of 10 nM (10^{-8} M); Tonon, Leroux, Leboulenger *et al.*, 1980; Verber van Kemenade, Jenks, Visser *et al.*, 1987). In mammals TRH stimulates the release of thyroid stimulating-hormone (TSH) (Folkers, Chang, Currie *et al.*, 1970), and prolactin (Rivier & Vale, 1974) from the PD, but in non-mammalian vertebrates TRH has no TSH-releasing activity (for review see Jackson, 1980). Instead, TRH has been shown to stimulate the release of prolactin

from the bullfrog PD *in vitro* (Clemons, Russell & Nicoll, 1979) and the release of α-MSH from the frog NIL (Tonon, Leroux, Stoeckel *et al.*, 1983a; for review see Tonon, 1984). The current demonstration of TRH-stimulation of α-MSH release from the goldfish pituitary provides evidence for the direct action of TRH on α-MSH release in teleosts.

There is little histochemical information regarding the existence or distribution of TRH-containing neurons in the brain or pituitary of vertebrates, or teleosts in particular (for reviews see Crim & Vigna, 1983; Jackson, 1980; Peter, 1986). Significant quantities of TRH are contained in the rat hypothalamus (Oliver, Eskay, Ben-Jonathan & Porter, 1974) and in the frog NIL (Giraud, Gillioz, Cont-Devolx & Oliver, 1979; Tonon, Leroux, Oliver *et al.*, 1983b; Seki, Nakai, Shioda *et al.*, 1980). As well, TRH has been detected in the brain, retina and especially the skin of several amphibians (Crim & Vigna, 1983); TRH-immunoreactivity in various parts of the frog and tadpole brain and pituitary has also been described (Mimnagh, Bolaffi, Montgomery & Kaltenbach, 1987). Jackson & Reichlin (1980) previously demonstrated the presence of TRH (100-200 pg/mg) in the pituitary and hypothalamus of a salmon (*Salmo sebago*). TRH has also been detected in the brains of hagfish, 2 species of sharks, and the retina of an eel (Crim & Vigna, 1983).

In this study multiple treatment of NIL fragments with TRH caused consistent α-MSH release responses; α-MSH returned to basal levels following each response to TRH. Under these conditions, dopamine inhibited total α-MSH release with an ED_{50} of 19.6 nM ($10^{-7.7}$ M); this value is comparable with the ED_{50} value of 61 nM ($10^{-7.2}$ M) for dopamine inhibition of spontaneous α-MSH release. In comparison, the ED_{50} values for dopamine inhibition of GtH release from PD fragments repeatedly

treated with sGnRH and sGnRH-A were 32 and 350 nM ($10^{-7.5}$, and $10^{-6.5}$ M), respectively; the ED_{50} for dopamine inhibition of spontaneous GtH release was 38 uM ($10^{-4.4}$ M). Data from a mammalian study indicates that dopamine inhibited α-MSH release from dispersed rat pituitary cells with an ED_{50} of 32 nM ($10^{-7.5}$ M) (Munemura, Cote, Tsuruta et al., 1980).

Apomorphine, a dopamine agonist, has been used to study dopamine receptors in the pituitary and brain in several vertebrate species. Our data indicate that (-)-apomorphine is more consistent and somewhat more active than (+)-apomorphine in inhibiting GtH and α-MSH release from the goldfish pituitary in vitro. Both stereoisomers of apomorphine modulated the effect of sGnRH and sGnRH-A. Also, both stereoisomers reduced total α-MSH release; however, only (-)-apomorphine abolished the α-MSH release responses to TRH. These data suggest subtle differences in the dopamine-mediated inhibition of spontaneous and releasing factor-stimulated release of GtH and α-MSH. In amphibia apomorphine is a potent inhibitor of spontaneous α-MSH release from dispersed pars intermedia cells in vitro (Tonon, Leroux, Stoeckel et al., 1983a). In the rat, (+)- and (-)-apomorphine were equipotent with dopamine in inhibiting α-MSH release from dispersed intermediate lobe cells (Munemura et al., 1980). However, (+)- and (-)-apomorphine have been shown to interact differentially with brain dopamine receptors; (-)-apomorphine injected into mice induced stereotypical cage-climbing behaviour, associated with stimulation of brain dopamine D2 receptors, and (+)-apomorphine strongly antagonized the action of (-)-apomorphine (Riffee, Wilcox, Smith et al., 1982). As our data demonstrate only weak stereoselectivity, I suggest that the dopamine receptor involved with

inhibition of GtH and α-MSH release differs somewhat from the classical dopamine D2 receptor subtype.

This is the first study to demonstrate that domperidone potently antagonizes the inhibitory actions of dopamine on GtH or α-MSH release from the teleost pituitary *in vitro*. In the present study, PD fragments were treated with sGnRH and sGnRH-A, while NIL fragments were treated with TRH; dopamine (500 nM, $10^{-6.3}$ M) inhibited total hormone release and abolished the GtH release-response to sGnRH and sGnRH-A. Domperidone reversed dopamine action and restored total GtH release as well as the GtH release-response to sGnRH; however, the GtH release-response to sGnRH-A was not restored by domperidone. This anomaly may stem from potential differences in the binding and catalytic properties of sGnRH and sGnRH-A; sGnRH-A has high receptor affinity which may cause functional changes in its receptor (H.R. Habibi, personal communication) and its long action may be due to its relative resistance to degradation. Domperidone also antagonized dopamine action and restored the α-MSH release-response to TRH and increased total α-MSH release in the presence of dopamine. Dopamine antagonists have been used previously to potentiate TRH action; haloperidol potentiated TRH stimulation of α-MSH release from amphibian intermediate lobe fragments *in vitro* (Tonon *et al.*, 1983a).

The present demonstraton of micromolar activity of domperidone in reversing dopamine action is in accord with *in vivo* data; domperidone at doses of umol/kg body weight (BW) injected i.p. into goldfish increased serum concentrations of GtH (this thesis, Chapter II). In comparison, sGnRH-A injected i.p. into goldfish was effective at nmol/kg BW doses. Additional evidence for a goldfish pituitary dopamine/neuroleptic

receptor comes from *in vivo* studies in which i.p.-injected [^{3}H]-domperidone was heavily concentrated by the pituitary. These results suggest that domperidone exerts its effects by binding to gonadotroph dopamine receptors, thereby blocking endogenous dopamine inhibition; it is also possible that domperidone may act presynaptically on dopamine and/or GnRH neurons in the pituitary to modify their activity. These results also suggest that the goldfish dopamine receptor has micromolar affinity for dopamine and dopamine ligands. In comparison, the binding site in the goldfish PD and NIL for [^{3}H]-spiperone has properties consistent with those of a receptor and has micromolar affinity for domperidone, pimozide and other dopamine ligands (this thesis, Chapter IV)

Historically, the classification of dopamine receptors has been controversial; presently, dopamine receptors are classified as either D1 or D2 subtypes (Leff & Creese, 1984). Only the D2 receptor subtype is sensitive to substituted benzamides and can discriminate between the active and inactive stereoisomers (-)- and (+)-sulpiride, respectively (Grigoriadis & Seeman, 1984). In the mammalian pituitary only the D2 receptor subtype is present. It has been shown that dopamine interacts with D2 receptors on lactotrophs and thyrotrophs in the anterior pituitary to inhibit prolactin and thyrotropin secretion, respectively (Foord, Peters, Dieguez *et al.*, 1983); as well, D2 receptors on melanotrophs in the posterior pituitary mediate dopamine inhibition of α-MSH release (for review see Cote, Eskay, Frey *et al.*, 1982; Tilders, Berkenbosch & Smelik, 1985). The existence of dopamine D2 receptors in the intermediate lobe of an amphibian, *Xenopus sp.*, has also been suggested (Verberg van Kemanade, Tonon, Jenks & Vaudry, 1986).

Domperidone is a specific dopamine D2 receptor antagonist which associates with a single, homogeneous, non-interacting population of brain dopamine receptors; based on the inability of non-dopaminergic agents and the stereoselectivity of dopamine D2 receptor antagonists to inhibit [^{3}H]-spiperone binding to brain dopamine receptors, domperidone is regarded as having a very high specificity for dopamine D2 receptors (Baudry, Martres & Schwartz, 1979).

In summary, the findings of this study support and extend the concept of pituitary dopamine receptor-mediated inhibition of GtH release in goldfish. In addition we present new findings on the control of α-MSH release in goldfish. TRH is a potent stimulator of α-MSH release from the goldfish NIL; as well, we confirm that sGnRH and sGnRH-A are potent stimulators of GtH release from the PD. Significantly, apomorphine inhibition of GtH and α-MSH release appears complex and only weakly stereoselective; stereoselectivity is one characteristic of the mammalian dopamine D2 receptor subtype. The present *in vitro* findings support our previous demonstration that domperidone increases GtH release *in vivo*. In conclusion, we propose that dopamine inhibition of GtH and α-MSH release from the goldfish pituitary *in vitro* is mediated by a common pituitary dopamine receptor which shares several characteristics with the mammalian dopamine D2 receptor.

Figure III-1. α-MSH RIA

Logit-log plot of displacement (B/B_o) in α-MSH RIA of α-MSH standards (●), neurointermediate lobe (NIL)-superfusate (▲), and pars distalis (PD)-superfusate (■). Values are means $\pm$ S.E.M. (n=5). Drawn lines are straight (r^2 = 0.968 to 0.998) and parallel (Parallel line test; Pekary, 1979).

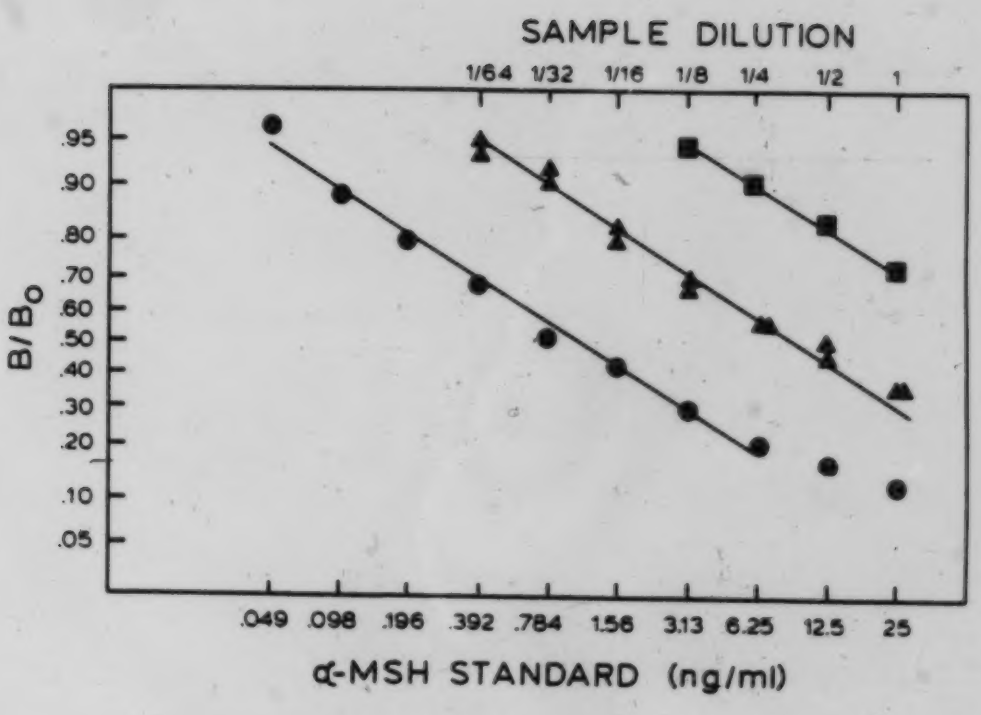
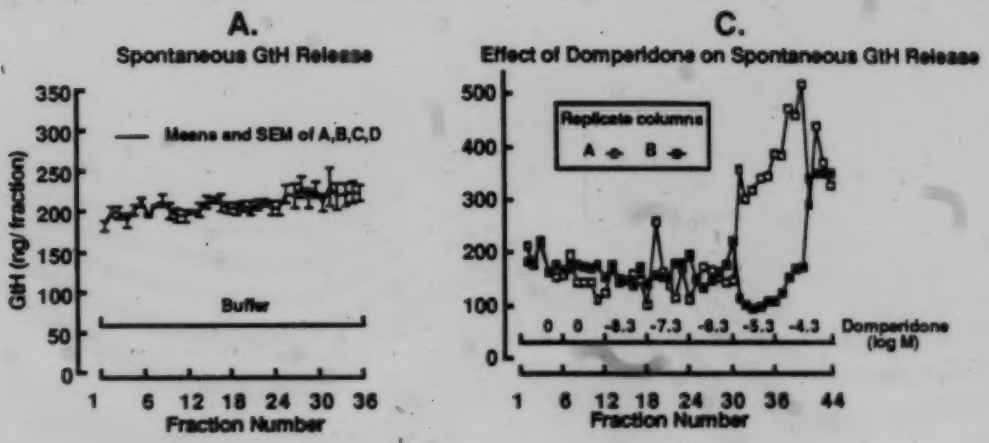

SAMPLE DILUTION
1/64 1/32 1/16 1/8 1/4 1/2 1
B/Bo
.95
.90
.80
.70
.60
.50
.40
.30
.20
.10
.05
.049 .098 .196 .392 .784 1.56 3.13 6.25 12.5 25
α-MSH STANDARD (ng/ml)

Figure III-2. Time course of the spontaneous release of GtH and dopamine inhibition of spontaneous GtH release.

GtH content (ng/fraction) of 10-min (2.5-ml) fractions collected from independent columns containing pars distalis fragments superfused with buffer alone (A), or a given concentration of dopamine (B), or domperidone (C). Values for fragments superfused with buffer alone (A) are means (± S.E.M.; n=4 columns); values for fragments superfused with dopamine (B) are single points from independent columns; values for domperidone-treated fragments are single points from independent columns plotted as a function of time.



B. Effect of Dopamine on Spontaneous GtH Release

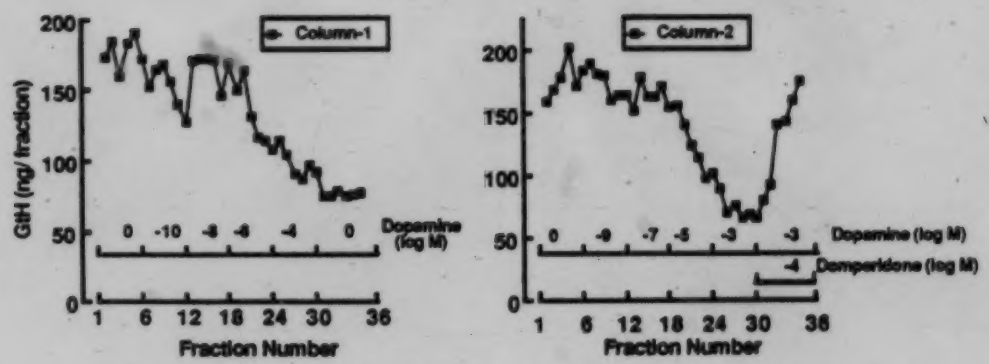


Figure III-3. ALLFIT analysis of dopamine inhibition of spontaneous GtH release from superfused pars distalis (PD) fragments.

ALLFIT plot of the mean GtH content of 6 X 10-min fractions collected from PD fragments superfused for 1 h periods with buffer (initial GtH release; 100 %) or a given concentration of dopamine (n= 2 to 4 columns for each point; raw data in Figure III-2B). Data for each column were normalized to express hormone content of fractions collected during each treatment as a percentage of the initial GtH release (first 60 min, buffer only) and plotted as a function of dopamine concentration. + indicates a significant ($p<0.05$) reduction in GtH content compared with initial GtH release.

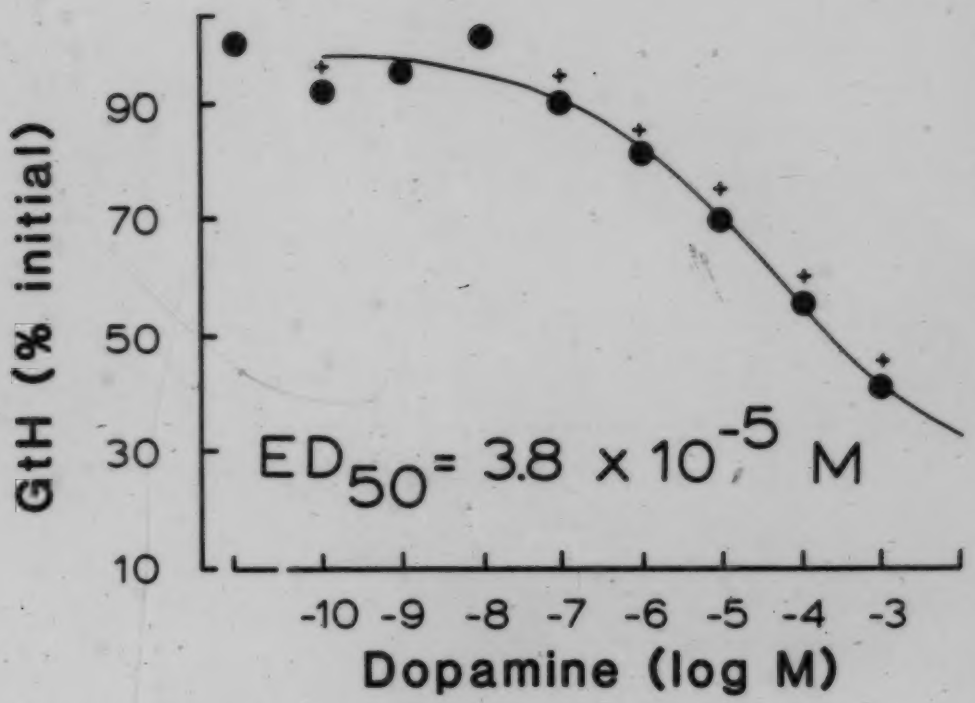

GtH (% initial)
90
70
50
30
10
$ED_{50} = 3.8 \times 10^{-5}$ M
-10 -9 -8 -7 -6 -5 -4 -3
Dopamine (log M)

Figure III-4. Time course of the spontaneous release of α-MSH and dopamine inhibition of spontaneous α-MSH release.

α-MSH content (ng/fraction) of 10-min (2.5-ml) fractions collected from columns containing neurointermediate lobe fragments superfused with buffer alone (A), or a given concentration of dopamine (B,C). Values for NIL fragments superfused with buffer alone (A) are means (± S.E.M.; n=4 columns); values for fragments treated with dopamine (B,C) are single points from independent columns plotted as a function of time.

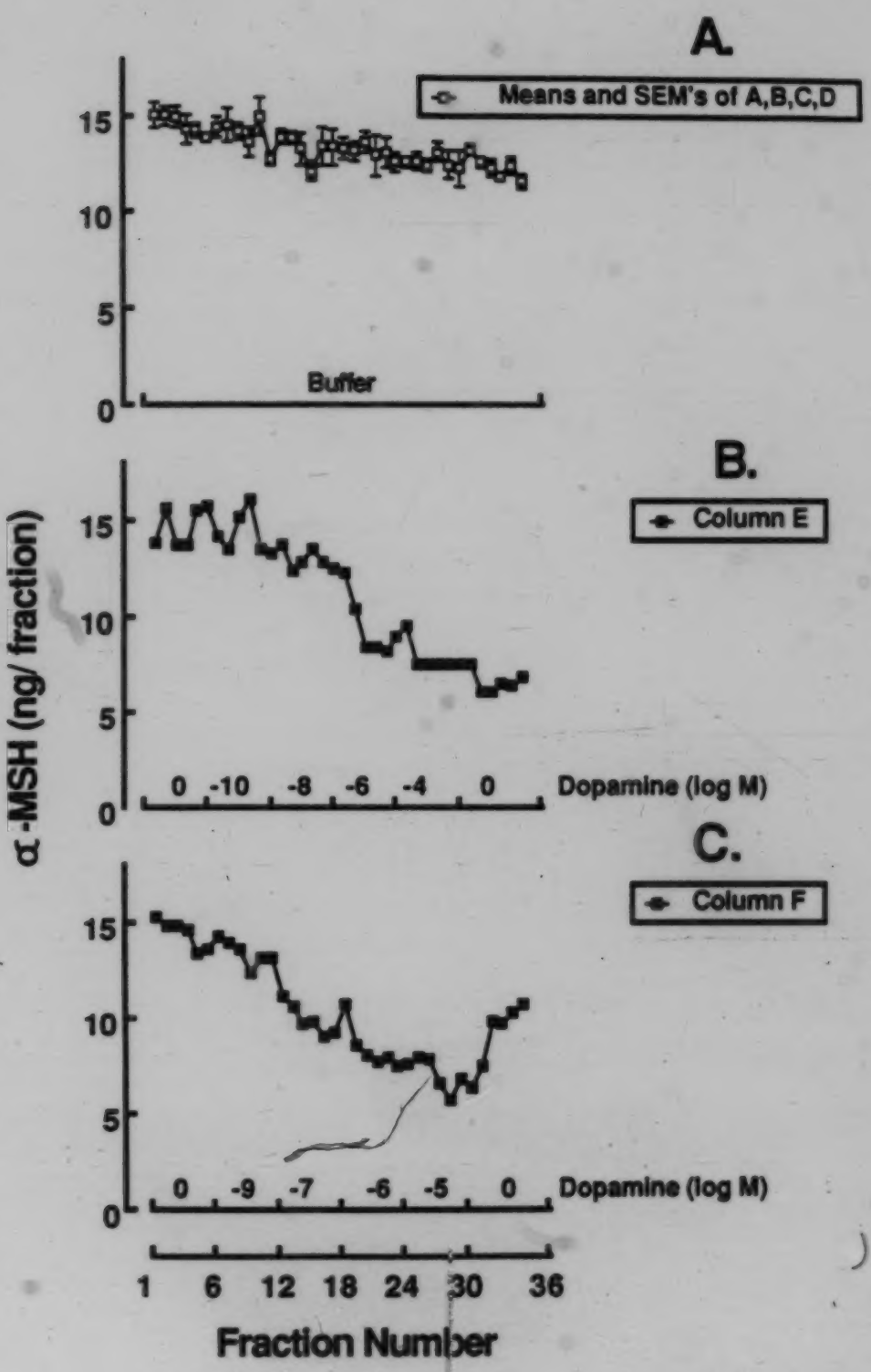

A.
Means and SEM's of A,B,C,D
15
10
5
0
Buffer
B.
Column E
15
10
5
0
0 -10 -8 -6 -4 0
Dopamine (log M)
α-MSH (ng/ fraction)
C.
Column F
15
10
5
0
0 -9 -7 -6 -5 0
Dopamine (log M)
1 6 12 18 24 30 36
Fraction Number

Figure III-5. ALLFIT analysis of dopamine inhibition of spontaneous α-MSH release from superfused neurointermediate lobe (NIL) fragments.

ALLFIT plot of the mean total α-MSH content of 6 X 10-min fractions collected from NIL fragments, from 2 independent columns, superfused with buffer (initial α-MSH release; 100 %) or a given concentration of dopamine (raw data in Figure III-4). Data for each column were normalized to express α-MSH content as a percentage of the initial α-MSH release (first 60 min, buffer only; 100 %) and plotted (values are means from 2 columns based on 6 fractions per columns) as a function of dopamine concentration. + indicates a significant ($p<0.05$) reduction in α-MSH content compared with initial α-MSH release .

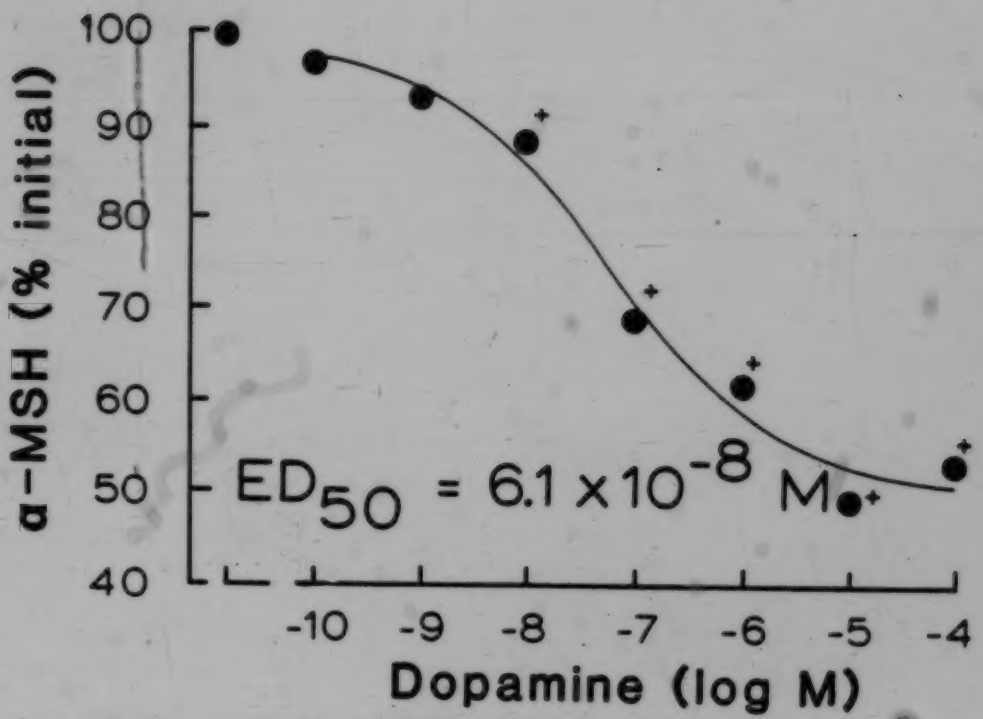

α-MSH (% initial)
100
90
80
70
60
50
40
$ED_{50} = 6.1 \times 10^{-8}$ M
-10
-9
-8
-7
-6
-5
-4
Dopamine (log M)

Figure III-6. TRH stimulation of α-MSH release.

α-MSH content (ng/fraction) of 10-min (2.5-ml) fractions collected from superfused neurointermediate lobe fragments in replicate columns (A, B) that received 3-min (0.75-ml) doses (↓) of pGlu-His-Pro-NH_2 (TRH; 10^{-10} to 10^{-5} M). Values are individual points from independent columns plotted as a function of time.

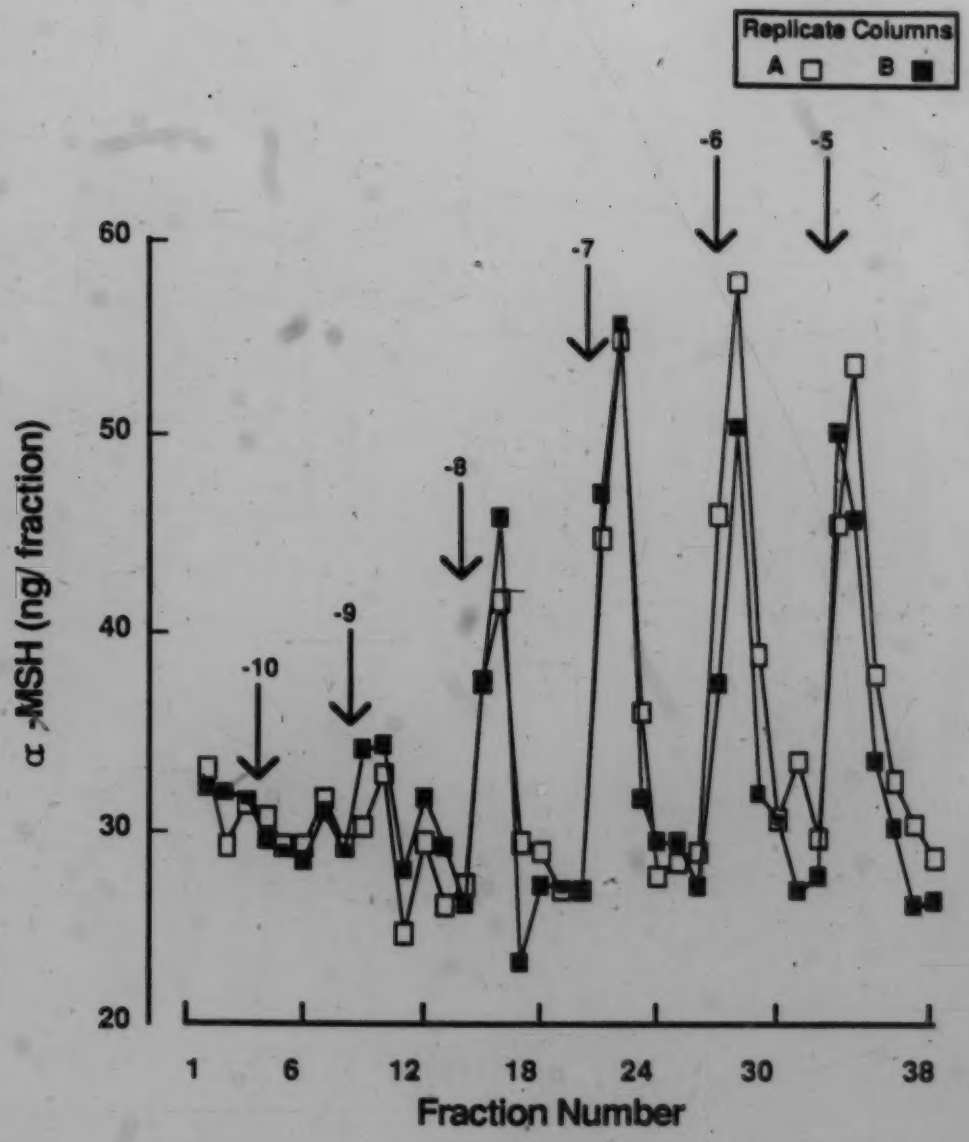

Replicate Columns
A
B
-10
-9
-8
-7
-6
-5
60
50
40
30
20
α -MSH (ng/ fraction)
1
6
12
18
24
30
38
Fraction Number

Figure III-7. ALLFIT analysis of TRH stimulation of α-MSH release.

ALLFIT plot of stimulated α-MSH release (α-MSH released in excess of pre-TRH levels in 4 X 10-min fractions including and following TRH stimulation; pre-TRH levels were estimated as the mean α-MSH content of 2 fractions preceding the TRH pulse) as a function of dopamine concentration (raw data in Figure III-6). + indicate values significantly different compared with values in the absence of TRH.

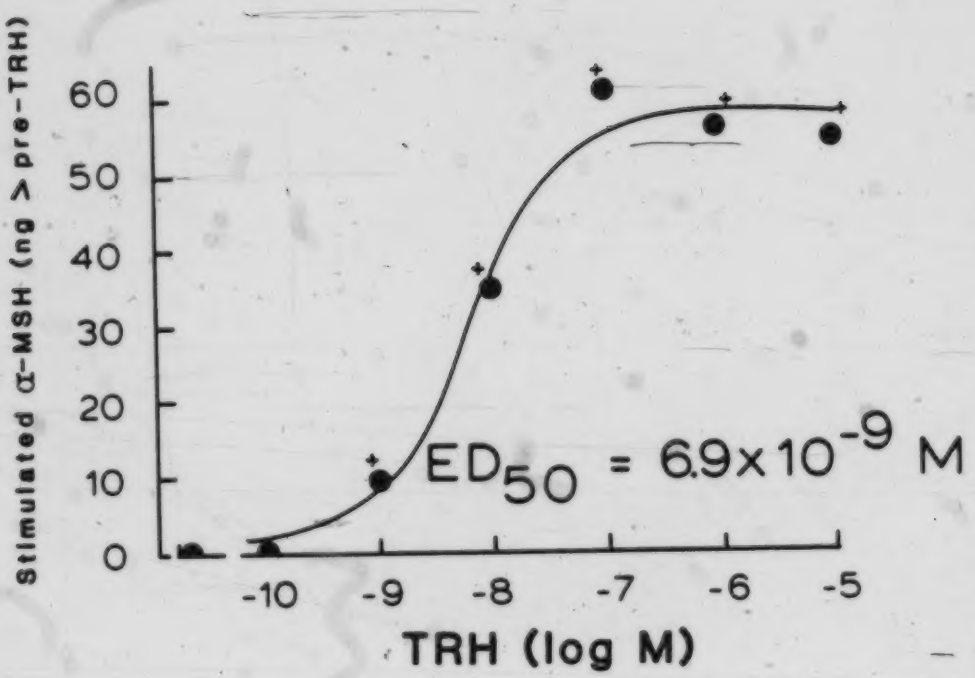

Stimulated α-MSH (ng > pre-TRH)
60
50
40
30
20
10
0
-10
-9
-8
-7
-6
-5
TRH (log M)
ED50 = 6.9x10-9 M

Figure III-8. sGnRH stimulation of GtH release and dopamine inhibition of GtH release from pars distalis (PD) fragments treated with [Trp^7, Leu^8]-LHRH (sGnRH).

GtH content (ng/fraction) of 10-min (2.5-ml) fractions collected from PD fragments that received multiple 3-min (0.75-ml) doses of sGnRH (20 nM, ↓) and were superfused with buffer or a given concentration of dopamine. Values are single points from individual columns plotted as a function of time.

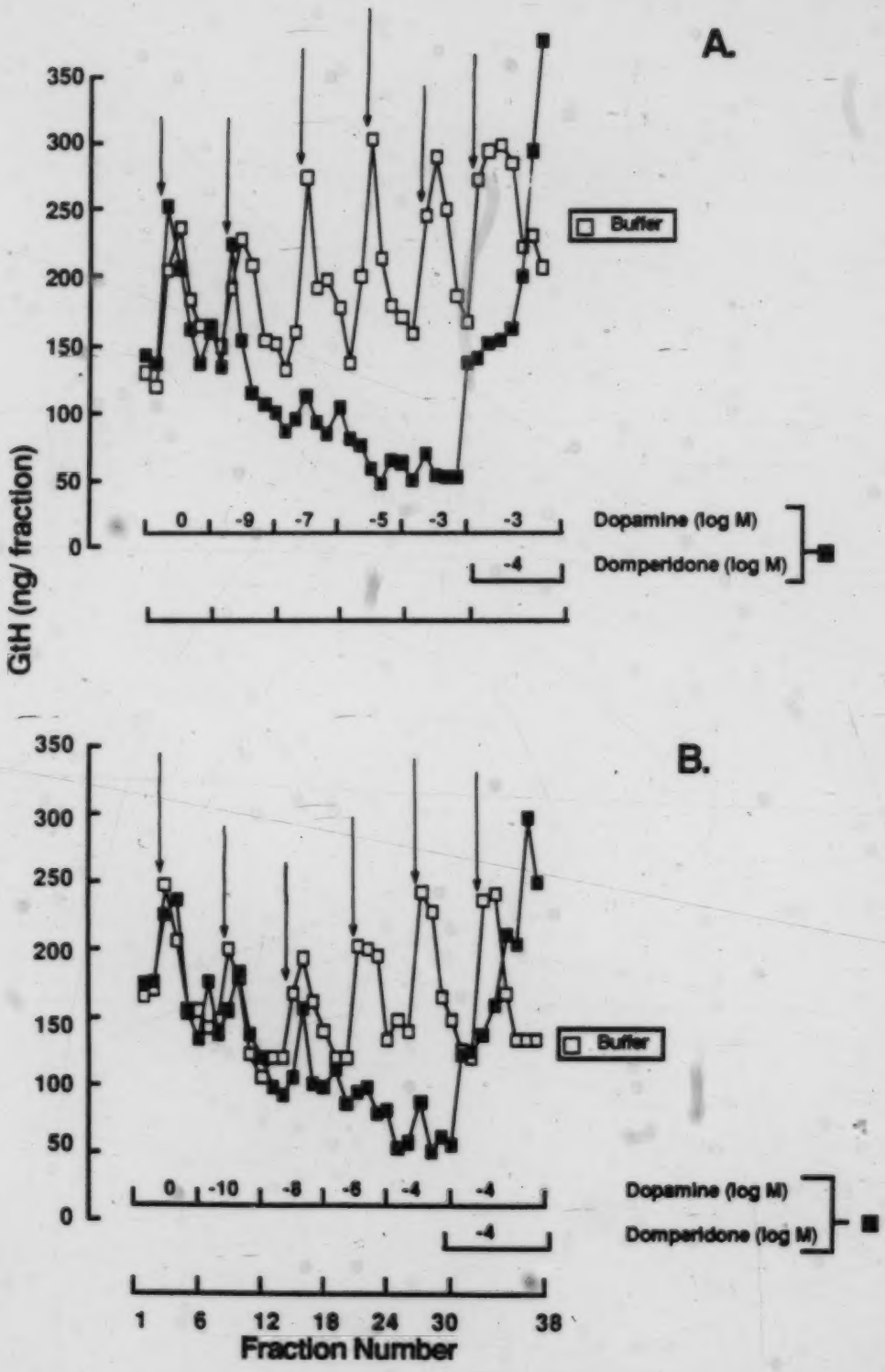

A.
350
300
250
200
150
100
50
0
GtH (ng/ fraction)
Buffer
0
-9
-7
-5
-3
-3
-4
Dopamine (log M)
Domperidone (log M)
B.
350
300
250
200
150
100
50
0
Buffer
0
-10
-8
-6
-4
-4
-4
Dopamine (log M)
Domperidone (log M)
1
6
12
18
24
30
38
Fraction Number

Figure III-9. ALLFIT analysis of dopamine inhibition of GtH release from pars distalis (PD) fragments treated with [Trp^7, Leu^8]-LHRH (sGnRH).

The GtH contents of 6 X 10-min fractions collected from PD fragments treated in trial periods with sGnRH and superfused with buffer of a given concentration of dopamine (2 fractions preceding, and 4 fractions including and following the sGnRH dose, 20 nM, 3-min, 0.75 ml; raw data in Figure III-8) were averaged and expressed as a percentage (% initial) of GtH released during the initial trial with buffer (initial release; 100 %); these mean values (based on 6 fractions each) were averaged among replicate columns (n=2 columns for each dopamine concentration; n=4 columns in the absence of dopamine; raw data in Figure III-8) and plotted (12 or 24 fractions) as a function of dopamine concentration. + indicates a significant ($p < 0.05$) reduction in GtH content compared with initial GtH release.

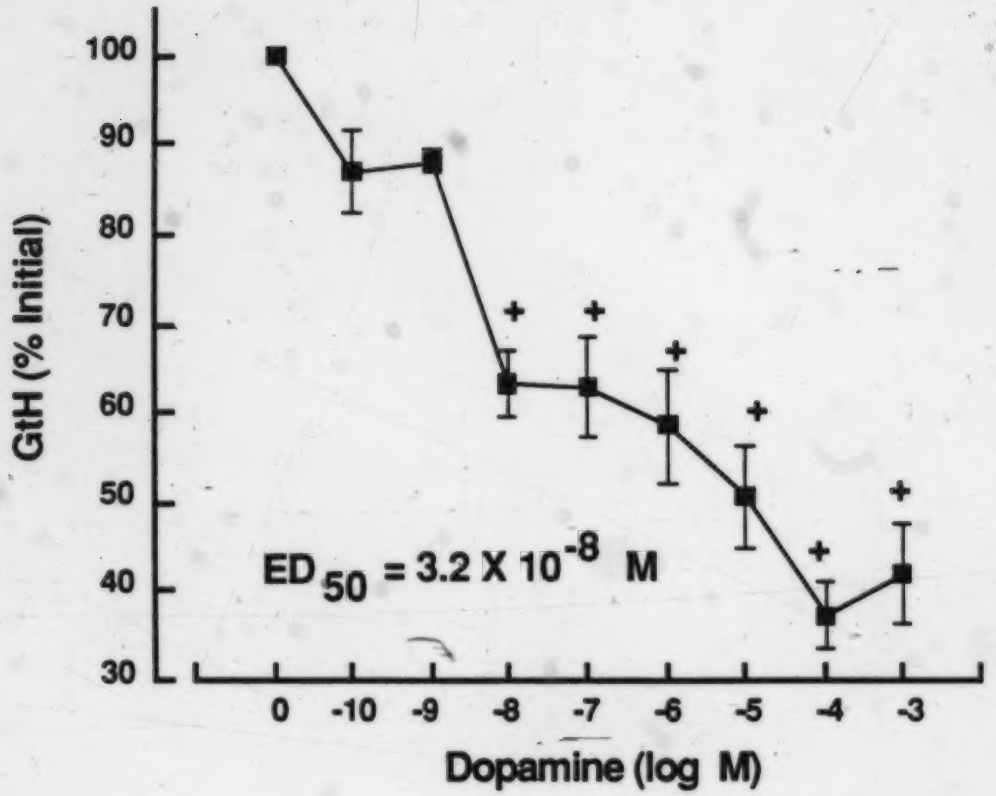

GtH (% Initial)
100
90
80
70
60
50
40
30
ED50 = 3.2 X 10-8 M
0
-10
-9
-8
-7
-6
-5
-4
-3
Dopamine (log M)

Figure III-10. sGnRH-A stimulation of GtH release and dopamine inhibition of GtH release from pars distalis (PD) fragments treated with sGnRH-A.

GtH content (ng/fraction) of 10-min (2.5-ml) fractions collected from PD fragments that received multiple 3-min (0.75-ml) doses of [DArg^{6}, Trp^{7}, Leu^{8}, Pro^{9}-N-ethylamide]-LHRH (sGnRH-A, 20 nM, ↓) and were superfused with buffer or a given concentration of dopamine. Values are single points from individual columns plotted as a function of time.

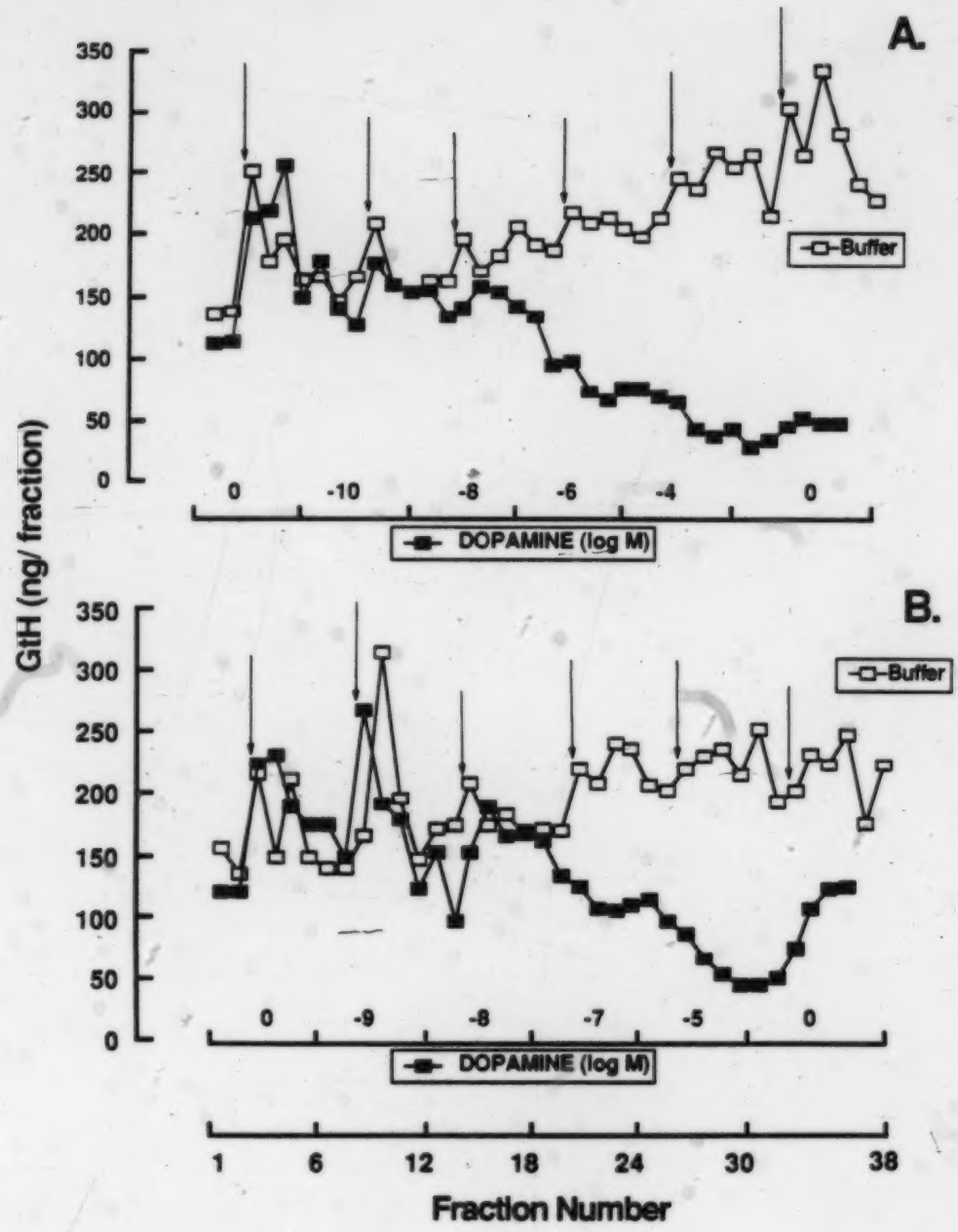

A.
B.
GtH (ng/ fraction)
350
300
250
200
150
100
50
0
Buffer
0
-10
-8
-6
-4
0
DOPAMINE (log M)
-9
-7
-5
1
6
12
18
24
30
38
Fraction Number

Figure III-11. ALLFIT analysis of dopamine inhibition of GtH release from pars distalis (PD) fragments treated with [DArg^{6}, Trp^{7}, Leu^{8}, Pro^{9}-N-ethylamide]-LHRH (sGnRH-A).

ALLFIT plot of the mean (± S.E.M.) GtH content of 6 X 10-min fractions collected from PD fragments from individual columns that received a 3-min (0.75-ml) dose of sGnRH-A (20 nM) and were superfused with buffer (initial GtH release; 100 %) or a given concentration of dopamine; data from each column were normalized to express GtH as a percentage of the initial GtH release (6 fractions) and plotted as a function of dopamine concentration (raw data in Figure III-10). + indicates a significant ($p<0.05$) reduction in GtH content compared with initial GtH release.

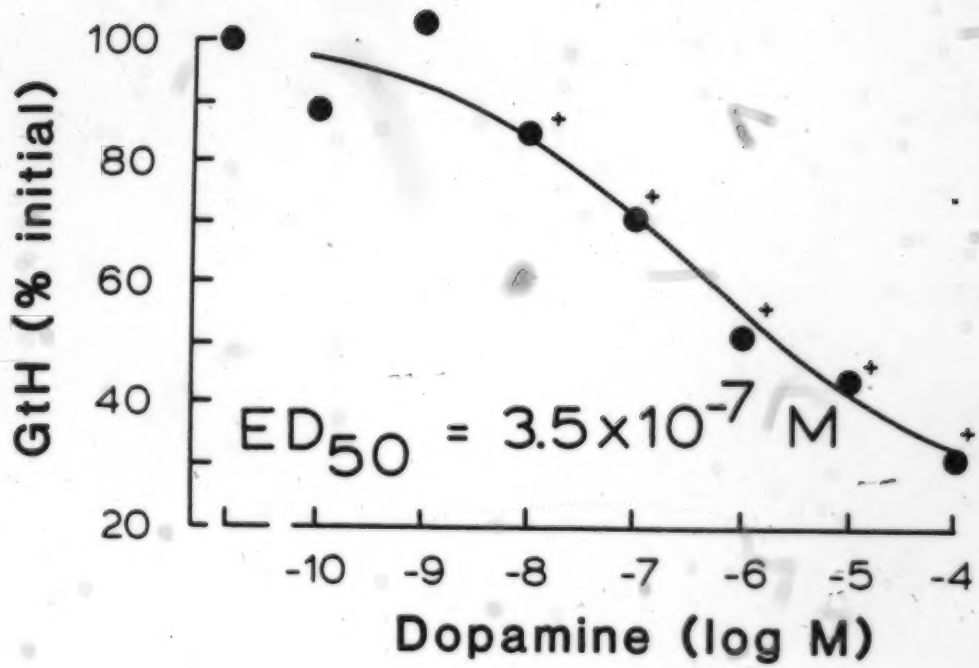

GtH (% initial)
100
80
60
40
20
ED50 = 3.5x10-7 M
-10
-9
-8
-7
-6
-5
-4
Dopamine (log M)

Figure III-12. TRH-stimulation of α-MSH release and dopamine inhibition of α-MSH release from neurointermediate lobe (NIL) fragments treated with TRH.

α-MSH content (ng/fraction) of 10-min (2.5-ml) fractions collected from superfused NIL fragments that received multiple 3-min (0.75-ml) doses of pGlu-His-Pro-NH_2 (TRH; 20 nM; ↓) and were superfused with buffer or a given concentration of dopamine. Values are single points from individual columns plotted as a function of time.

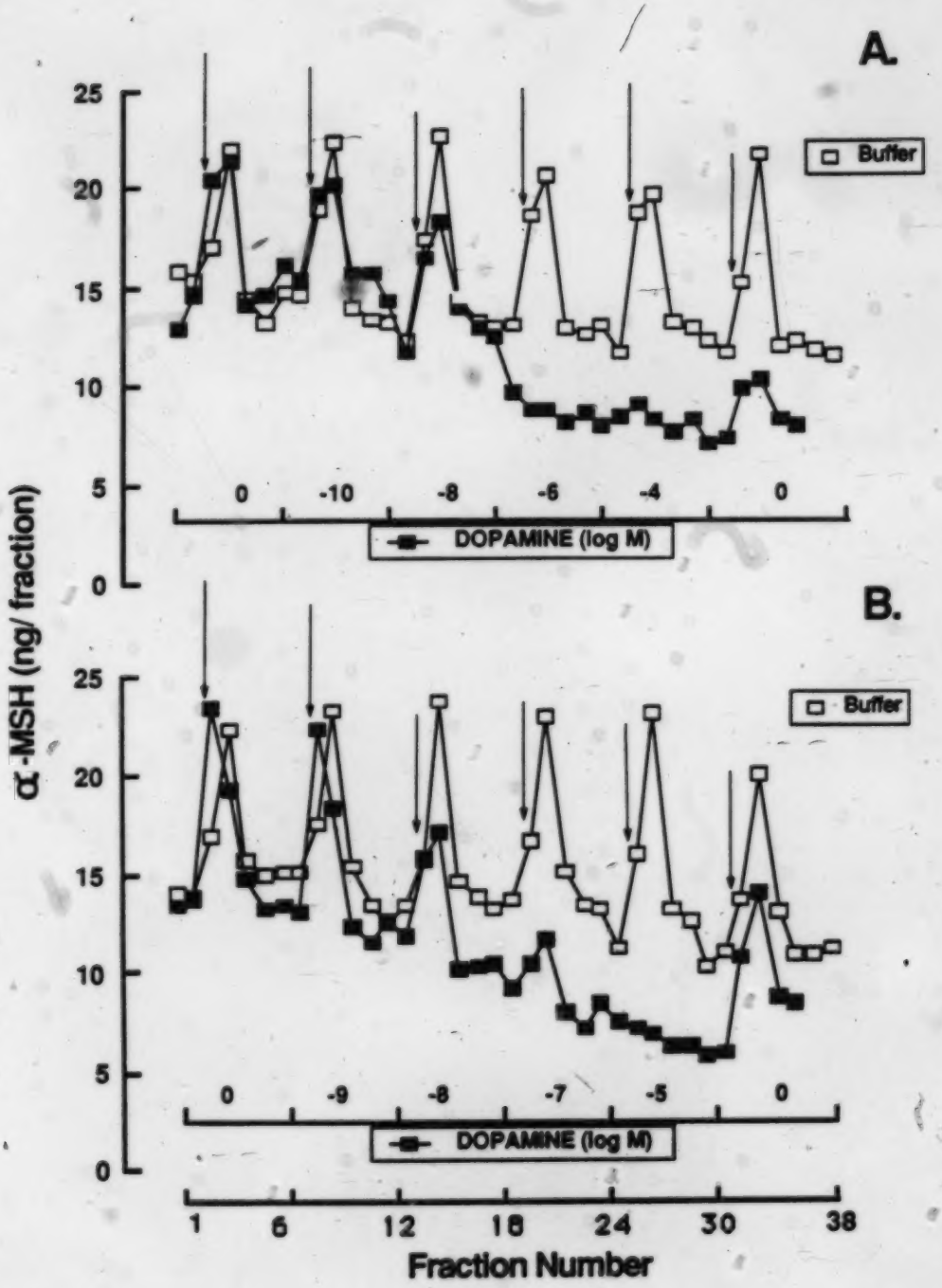

A.
Buffer
25
20
15
10
5
0
0
-10
-8
-6
-4
0
DOPAMINE (log M)
B.
Buffer
25
20
15
10
5
0
0
-9
-8
-7
-5
0
DOPAMINE (log M)
α-MSH (ng/ fraction)
1
6
12
18
24
30
38
Fraction Number

Figure III-13. ALLFIT analysis of dopamine inhibition of α-MSH release from neurointermediate lobe (NIL) fragments treated with TRH.

ALLFIT plot of the total α-MSH content of 6 X 10-min fractions collected from NIL fragments in 2 columns that received a 3-min (0.75-ml) dose of TRH (20 nM) and were superfused with buffer (initial α-MSH release; 100 %), or a given concentration of dopamine; data from each column were normalized to express α-MSH as a percentage of the initial α-MSH release (6 fractions) and plotted as a function of dopamine concentration (raw data in Figure III-13). + indicates a significant ($p<0.05$) decrease in α-MSH content compared with initial α-MSH release.

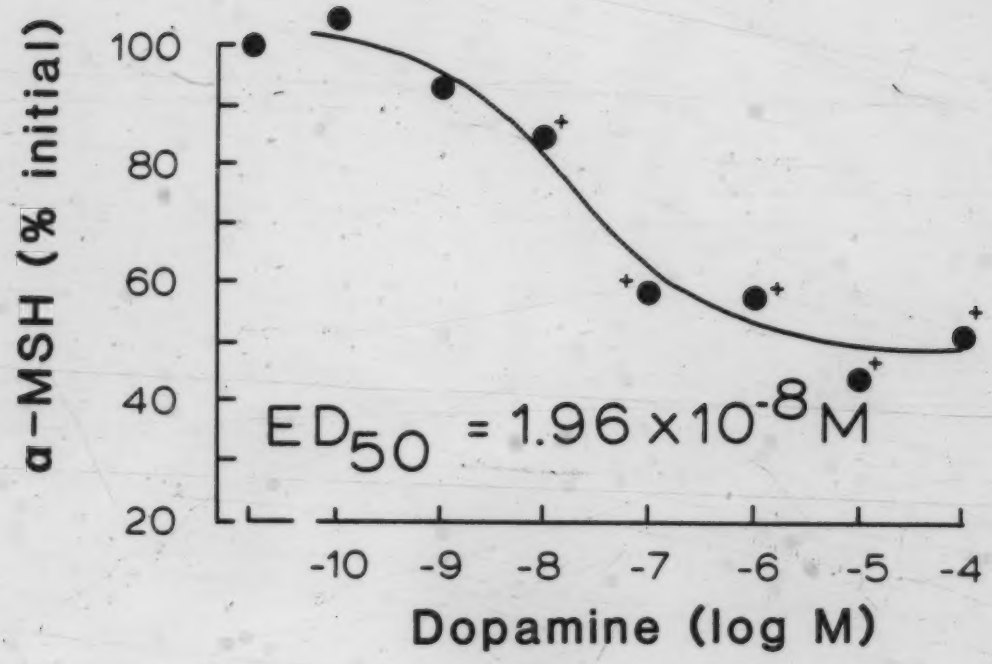

α-MSH (% initial)
100
80
60
40
20
ED50 = 1.96 x 10-8 M
-10
-9
-8
-7
-6
-5
-4
Dopamine (log M)

Figure III-14. Inhibition by (+)- and (-)-apomorphine of GtH release from pars distalis (PD) fragments treated with [Trp^7, Leu^8]-LHRH (sGnRH).

GtH content (ng/fraction) of 10-min (2.5-ml) fractions collected from PD- fragments that received multiple 3-min (0.75-ml) doses of sGnRH (20 nM, ↓) and were superfused with buffer or a given concentration of (+)-apomorphine (Figure III-14A) or (-)-apomorphine (Figure III-14B). Values are single points from replicate columns plotted as a function of time.

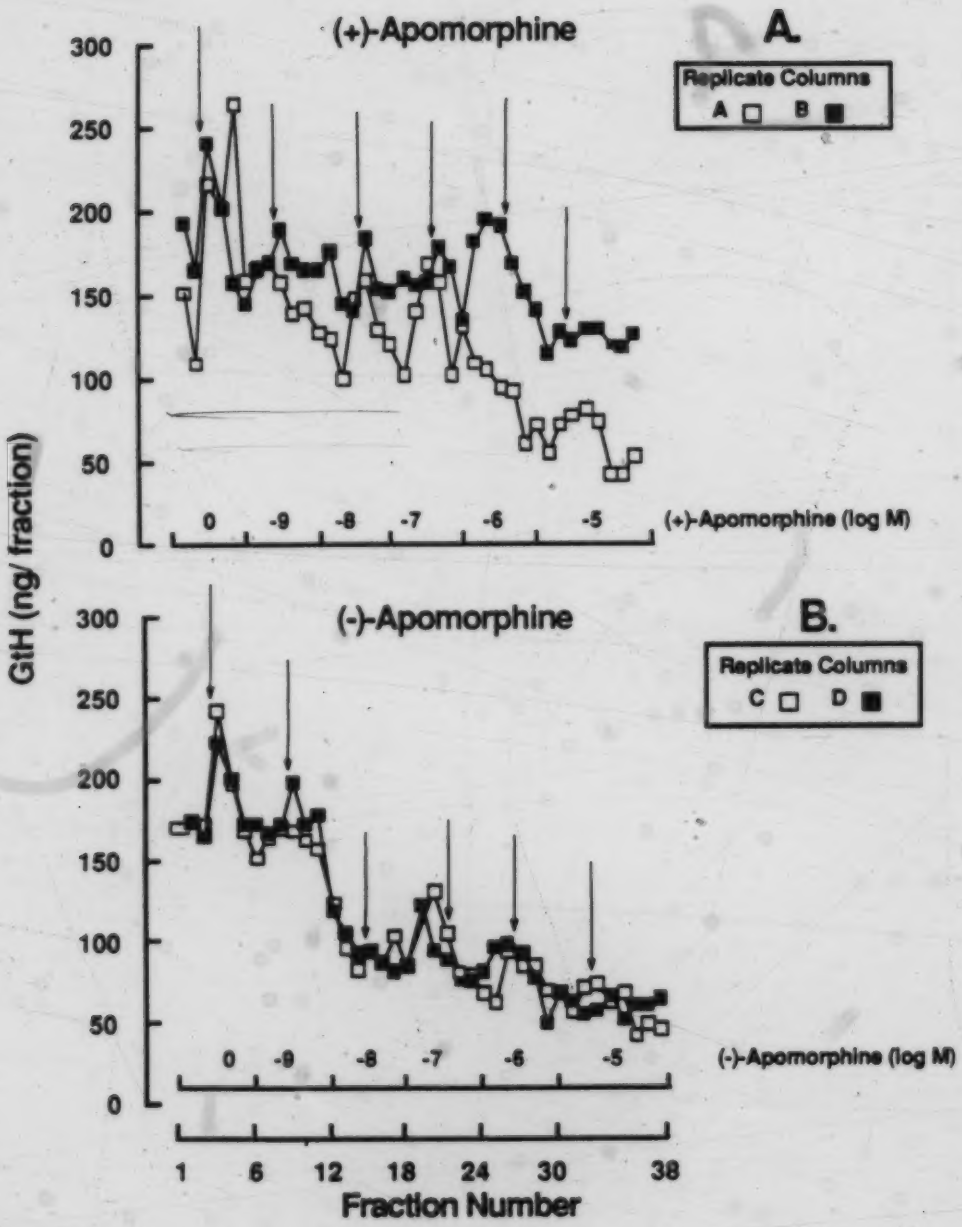

(+)-Apomorphine
A.
Replicate Columns
A □ B ■
300
250
200
150
100
50
0
0 -9 -8 -7 -6 -5
(+)-Apomorphine (log M)
GtH (ng/ fraction)
(-)-Apomorphine
B.
Replicate Columns
C □ D ■
0 -9 -8 -7 -6 -5
(-)-Apomorphine (log M)
1 6 12 18 24 30 38
Fraction Number

Figure III-15. ALLFIT analysis of apomorphine inhibition of GtH release from pars distalis (PD) fragments treated with [Trp^7, Leu^8]-LHRH (sGnRH).

The GtH contents of 6 X 10-min fractions collected from PD fragments treated in trial periods with sGnRH and superfused with buffer or a given concentration of (+)- or (-)-apomorphine (2 fractions preceding, and 4 fractions including and following the sGnRH dose, 20 nM, 3-min, 0.75 ml; raw data in Figure III-14) were averaged and expressed as a percentage (% initial) of GtH released during the initial trial with buffer (initial release; 100 %). Mean values (6 fractions per column) were averaged among replicate columns (n=2 columns for each apomorphine concentration; n=4 columns in absence of apomorphine) and plotted (12 or 24 columns) as a function of apomorphine concentration. + indicates a significant ($p < 0.05$) reduction in GtH content compared with initial GtH release; * indicates significant ($p < 0.05$) difference compared with a similar dose of the alternate enantiomer.

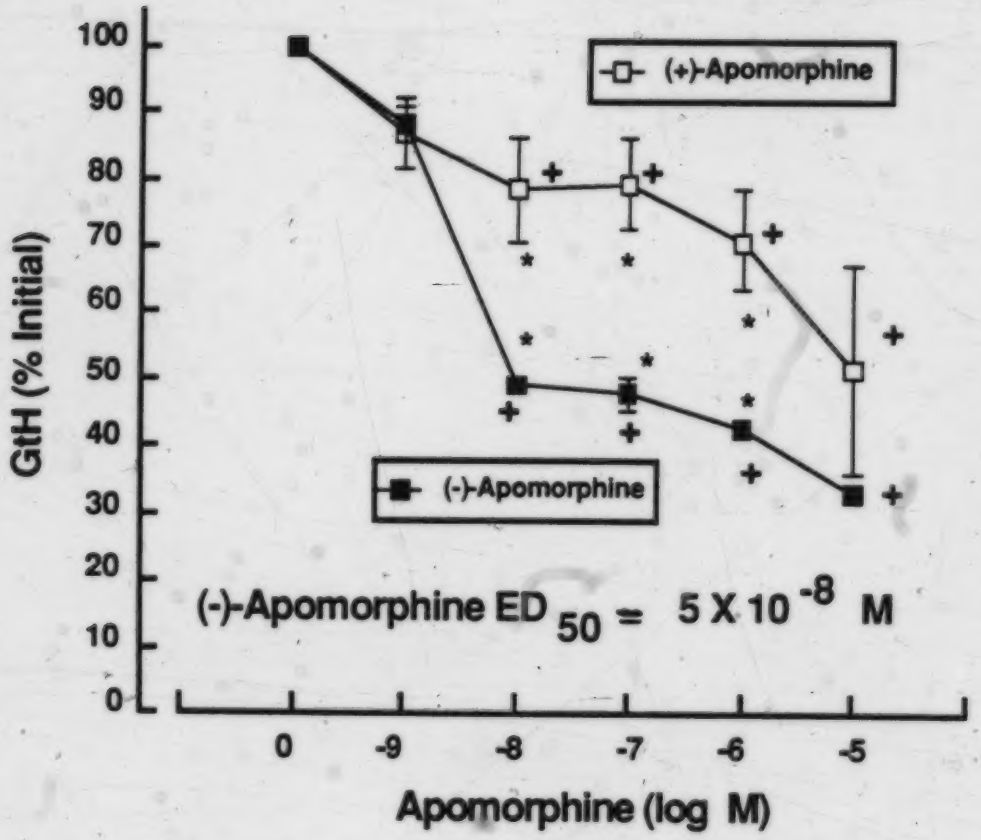

100
90
80
70
60
50
40
30
20
10
0
GtH (% Initial)
(+)-Apomorphine
(-)-Apomorphine
(-)-Apomorphine ED 50 = 5 X 10 -8 M
0
-9
-8
-7
-6
-5
Apomorphine (log M)

Figure III-16. Inhibition by (+)- or (-)-apomorphine of GtH and α -MSH release from pituitary fragments treated with releasing factors.

A. GtH content (ng/fraction) of 10-min (2.5-ml) fractions collected from superfused pars distalis fragments that received multiple 3-min (0.75 ml) doses of [DArg^6, Trp^7, Leu^8, Pro^9-N-ethylamide]-LHRH (sGnRH-A, 20 nM, ↓) (Figure III-16A).

B. α -MSH content (ng/fraction) of 10-min (2.5-ml) fractions collected from superfused neurointermediate lobe fragments that received multiple 3-min (0.75 ml) doses of pGlu-His-Pro-NH_2 (TRH; 20 nM) (Figure III-16B). Values are single points from individual columns plotted as a function of time.

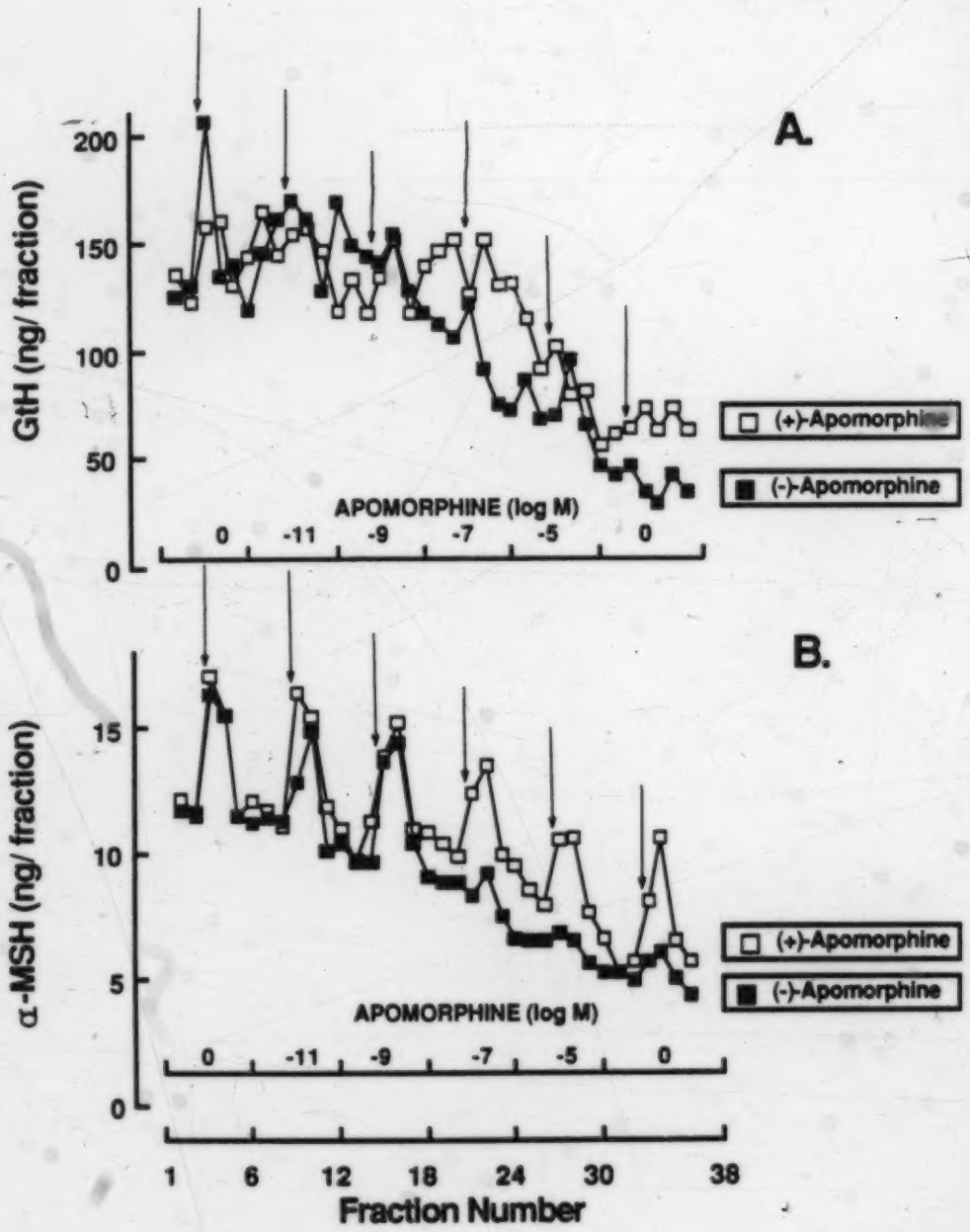

A.
GtH (ng/ fraction)
200
150
100
50
0
(+)-Apomorphine
(-)-Apomorphine
APOMORPHINE (log M)
0
-11
-9
-7
-5
0
B.
α-MSH (ng/ fraction)
15
10
5
0
(+)-Apomorphine
(-)-Apomorphine
APOMORPHINE (log M)
0
-11
-9
-7
-5
0
1
6
12
18
24
30
38
Fraction Number

Figure III-17. Effects of domperidone on GtH release from pars distalis fragments treated with [Trp^7, Leu^8]-LHRH (sGnRH) in the presence or absence of dopamine (500 nM).

GtH content (ng/fraction) of 10-min (2.5-ml) fractions collected from pars distalis fragments in independent columns that received a 3-min (0.75-ml) dose of sGnRH (20 nM, ↓) and were superfused with buffer or a given dose of domperidone, in the presence (Figure III-17A) or absence (Figure III-17B) of dopamine. Values are individual points from replicate columns plotted as a function of time.

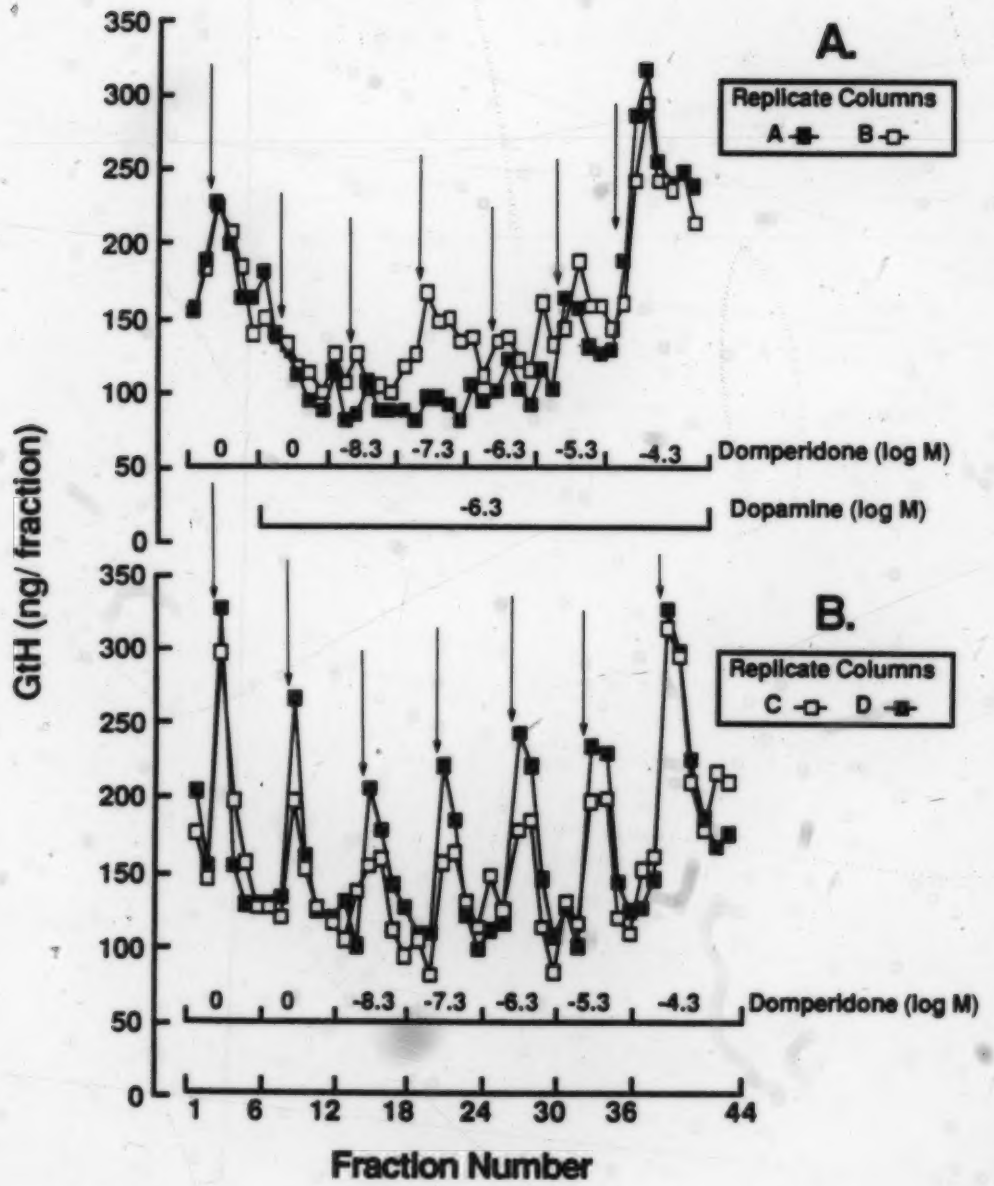

A.
Replicate Columns
A
B
B.
Replicate Columns
C
D
GtH (ng/ fraction)
350
300
250
200
150
100
50
0
0
0
-8.3
-7.3
-6.3
-5.3
-4.3
Domperidone (log M)
-6.3
Dopamine (log M)
1
6
12
18
24
30
36
44
Fraction Number

Figure III-18. Analysis of domperidone effects on GtH release from pars distalis (PD) fragments treated with [Trp^7, Leu^8]-LHRH (sGnRH) in the presence or absence of dopamine (500 nM).

The GtH contents of 6 X 10-min fractions collected from PD fragments treated in trial periods with sGnRH and superfused with a given concentration of domperidone in the presence or absence of dopamine (2 fractions preceding, and 4 fractions including and following the sGnRH dose, 20 nM, 3-min, 0.75 ml; raw data in Figure III-17) were averaged and expressed as a percentage of GtH released during the initial trial in the absence of domperidone (initial release; 100 %); these mean values (6 fractions per columns) were averaged among replicate columns (n=4 columns in the presence of dopamine; n=2 columns in the presence of dopamine) and plotted (24 or 12 columns) as a function of domperidone concentration. * indicates a significant ($p < 0.05$) reduction in GtH content compared with initial GtH release in the absence of domperidone.

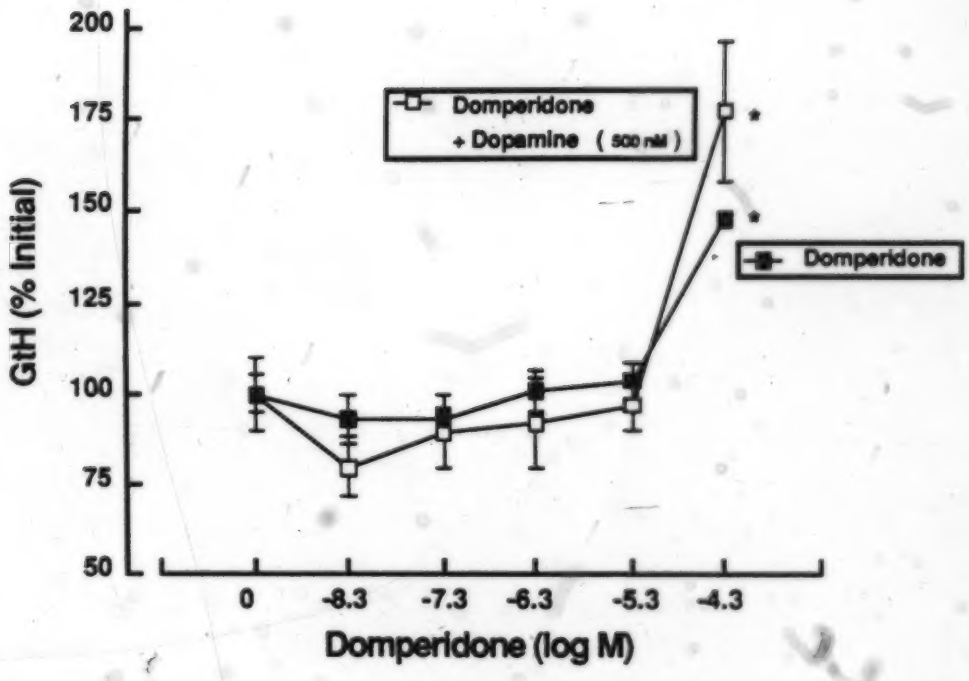

200
175
150
125
100
75
50
GtH (% Initial)
Domperidone
+ Dopamine (500 nM)
Domperidone
*
*
0
-8.3
-7.3
-6.3
-5.3
-4.3
Domperidone (log M)

Figure III-19. Domperidone antagonism of dopamine inhibition of GtH release from pars distalis (PD) fragments treated with sGnRH-A.

GtH content (ng/fraction) of 10-min (2.5-ml) fractions collected from pars distalis fragments in independent columns that received a 3-min (0.75-ml) dose of [DArg^{6}, Trp^{7}, Leu^{8}, Pro^{9}-N-ethylamide]-LHRH (sGnRH-A; 20 nM; ↓) and were superfused with buffer, or dopamine (500 nM) in the absence or presence of a given dose of domperidone. Values are individual points from independent superfusion columns plotted as a function of time.

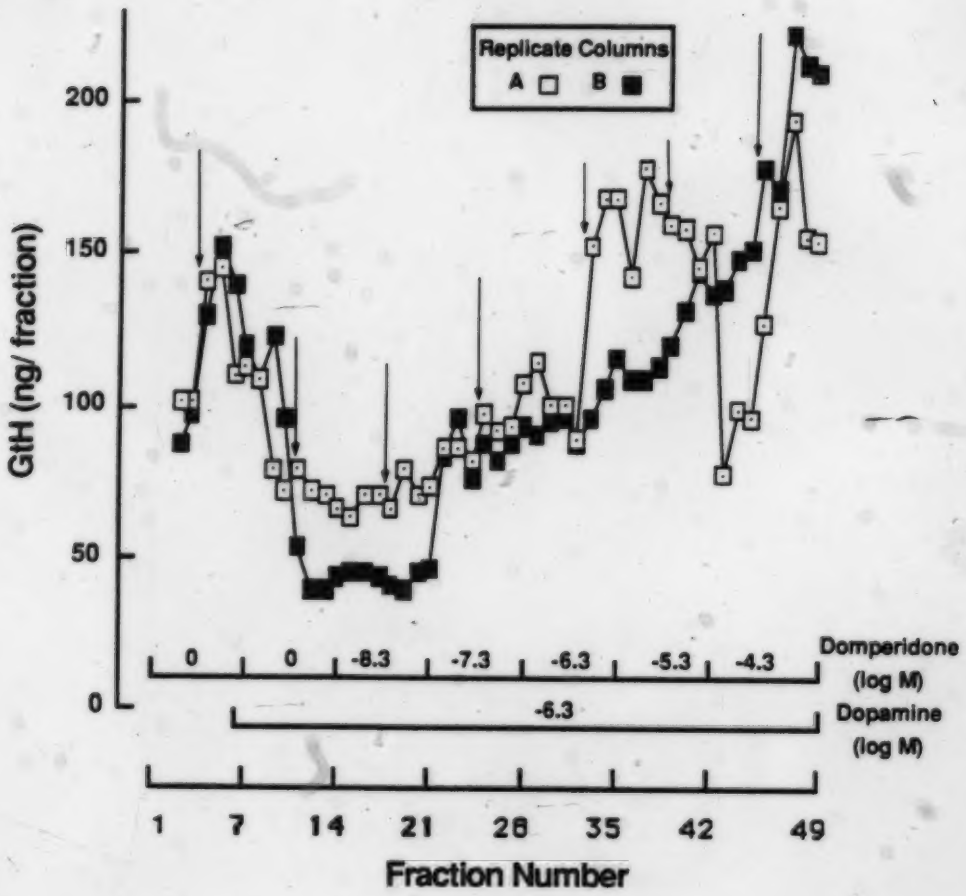

Replicate Columns
A
B
GtH (ng/ fraction)
200
150
100
50
0
0
0
-8.3
-7.3
-6.3
-5.3
-4.3
Domperidone (log M)
-6.3
Dopamine (log M)
1
7
14
21
28
35
42
49
Fraction Number

Figure III-20. ALLFIT analysis of domperidone effects on GtH release from pars distalis (PD) fragments treated with [DArg^{6}, Trp^{7}, Leu^{8}, Pro^{9}-N-ethylamide]-LHRH (sGnRH-A) and superfused with dopamine (500 nM).

The GtH contents of 6 X 10-min fractions collected from PD fragments treated in trial periods with sGnRH and superfused with a given concentration of domperidone in the presence of dopamine (2 fractions preceding, and 4 fractions including and following the sGnRH dose, 20 nM, 3-min, 0.75 ml; raw data in Figure III-19) were averaged and expressed as a percentage of GtH released during the initial trial in the absence of domperidone (initial release; 100 %); these mean values (6 fractions per column) were averaged among replicate columns (n=2 columns for each domperidone concentration; n=4 columns in the absence of domperidone) and plotted as a function of domperidone concentration. + indicates a significant ($p < 0.05$) reduction in GtH content compared with initial GtH release.

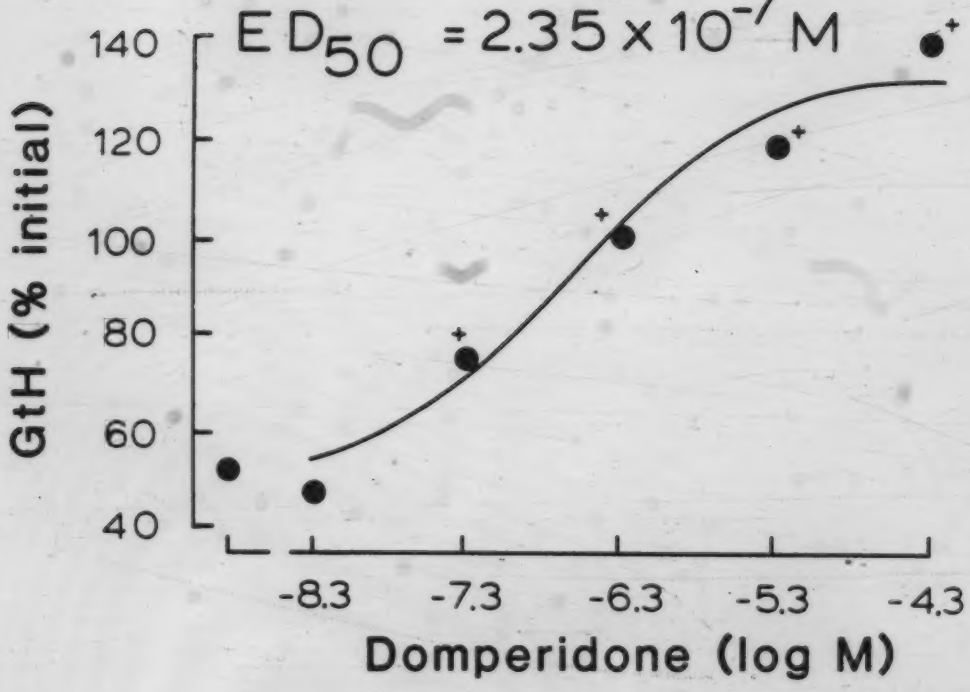

ED50 = 2.35 x 10-7 M
140
120
100
80
60
40
GtH (% initial)
-8.3
-7.3
-6.3
-5.3
-4.3
Domperidone (log M)

Figure III-21. Domperidone antagonism of dopamine inhibition of α-MSH release from neurointermediate lobe fragments treated with TRH.

α-MSH content (ng/fraction) of 10-min (2.5-ml) fractions collected from neurointermediate lobe fragments in independent columns that received a 3-min (0.75-ml) dose of pGlu-His-Pro-NH_2 (TRH; 20 nM; ↓) and were superfused with buffer, dopamine (500 nM), or dopamine and a given concentration of domperidone. Values are individual points from independent columns plotted as a function of time.

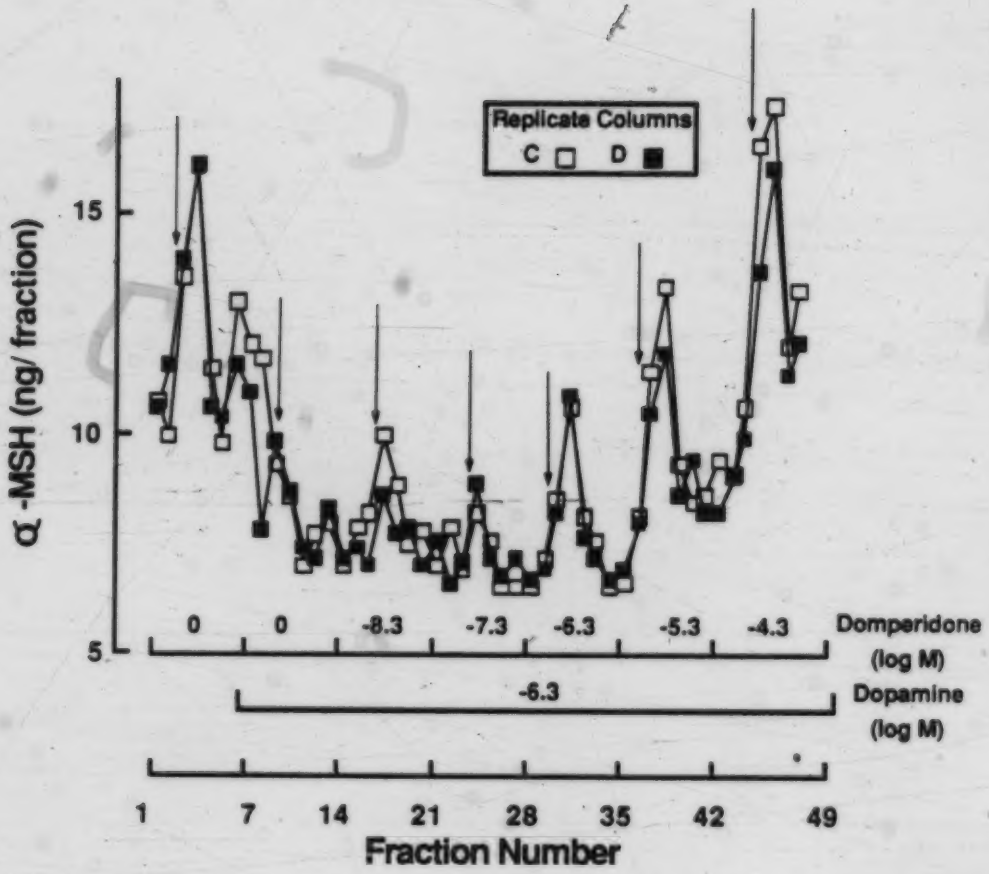
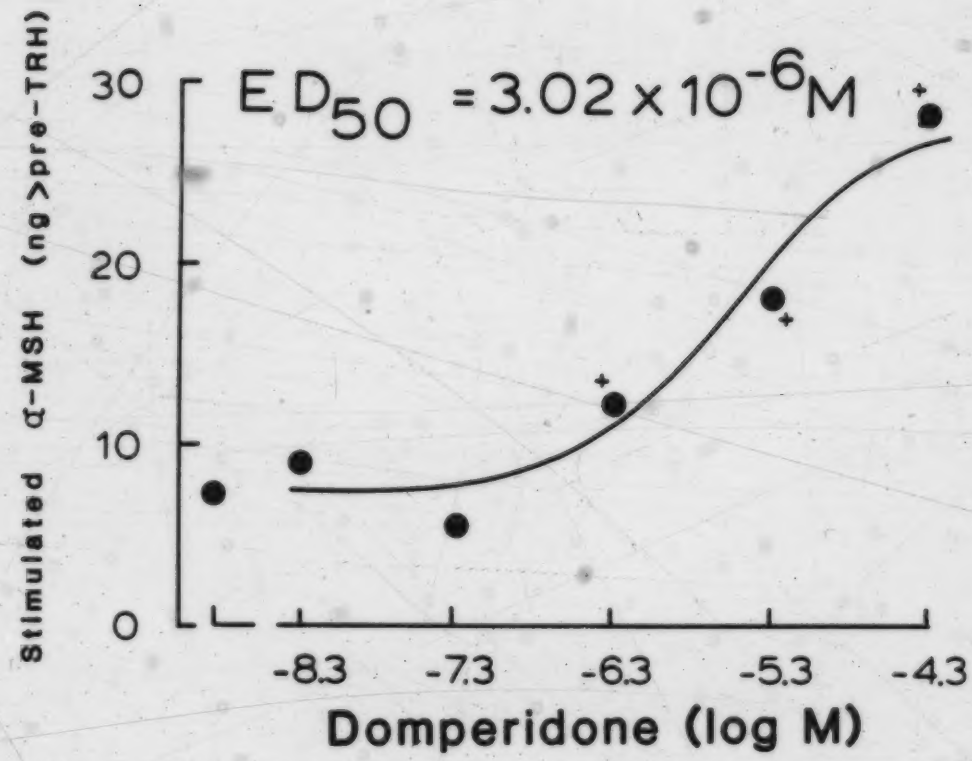

Replicate Columns
C
D
α -MSH (ng/ fraction)
15
10
5
0
0
-8.3
-7.3
-6.3
-5.3
-4.3
Domperidone (log M)
-6.3
Dopamine (log M)
1
7
14
21
28
35
42
49
Fraction Number

Figure III-22. ALLFIT analysis of the effect of domperidone on stimulated α-MSH release by pGlu-His-Pro-NH_2 (TRH) from neurointermediate lobe (NIL) fragments in the presence of dopamine (500 nM).

ALLFIT plot of stimulated α-MSH release (α-MSH released in excess of pre-TRH levels from the 4 fractions including and following each dose of TRH; pre-TRH levels were estimated as the mean α-MSH content of 2 fractions preceding the dose of TRH) as a function of domperidone concentration (raw data in Figure III-21). Values are mean ($\pm$ S.E.M.) stimulated α-MSH release from duplicate columns. + indicate values significantly ($p < 0.05$, t-test) greater than those observed during superfusion with dopamine (500 nM) alone.


ED50 = 3.02 x 10-6M
Stimulated α-MSH (ng >pre-TRH)
30
20
10
0
-8.3
-7.3
-6.3
-5.3
-4.3
Domperidone (log M)

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IV. *In vitro* binding characteristics of [^{3}H]-spiperone to the goldfish (*Carassius auratus*) pituitary.

INTRODUCTION

In a wide variety of teleost fishes evidence indicates that dopamine inhibits the release of gonadotropic hormone (GtH) by acting directly on the pituitary to modulate the action of gonadotropin-releasing hormone (GnRH) (reviewed by Peter, Chang, Nahorniak *et al.*, 1986). In addition, dopamine is the only endogenous neurotransmitter shown to inhibit spontaneous GtH release *in vivo* (Chang, Cook & Peter, 1983; Chang, Peter, Nahorniak & Sokolowska, 1984a; Chang & Peter, 1983) and *in vitro* (Chang, MacKenzie, Gould & Peter, 1984b; this thesis, Chapter III) in goldfish (*Carassius auratus*). Dopamine has also been implicated as an inhibitor of the release of other teleost pituitary hormones including prolactin (Olivereau, 1975; reviewed by Peter & Fryer, 1983), and α-melanocyte stimulating hormone (α-MSH) (Olivereau, 1978; Olivereau, Olivereau & Lambert, 1987; this thesis, Chapter III).

In the mammalian pituitary it has been demonstrated that dopamine interacts with dopamine:D2 receptors on the lactotrophs of the anterior pituitary to inhibit the release of prolactin (Cronin, 1982), and on the melanotrophs of the intermediate lobe to inhibit the release of α-MSH (Cote, Eskay, Frey, Grewe, Munemura, Stoof, Tsuruta & Kebabian, 1982).

Recently, we presented *in vivo* evidence for specific dopamine-receptor mediated inhibition of GtH release in goldfish (this thesis Chapter II) and proposed that dopamine inhibits GtH release from the goldfish pituitary by stimulating a dopamine:D2-like receptor on the

gonadotroph. The purpose of the present research was to investigate the binding characteristics of a goldfish pituitary [^{3}H]-spiperone/neuroleptic binding site, which we propose represents the goldfish pituitary dopamine receptor associated with inhibition of GtH secretion.

MATERIALS AND METHODS

Experimental animals:

Common or comet variety goldfish (approximately 40 g; 13-15 cm) of mixed sex were obtained from Grassyforks Fisheries Co., Martinsville, Indiana or from Ozark Fisheries, Stoutland, Missouri. Fish were maintained for several weeks in flow-through aquaria at simulated ambient temperature (7-19 ^{o}C) and photoperiod (8-17 h photophase), and fed twice daily with commercial trout food. In all cases animals were anaesthetized (tricaine methanesulphonate; 0.5 g/l) prior to any handling. To harvest pituitary glands, fish were anaesthetized, killed by spinal transection just posterior to the medulla oblongata, and the pituitary removed and placed into ice-cold assay buffer (Tris, 50 mM; NaCl 120 mM; KCl, 5 mM; $MgCl_2$, 1 mM; $CaCl_2$, 2 mM; pH=7.4). All subsequent procedures were performed at 0-4 ^{o}C.

[^{3}H]-Spiperone Radioreceptor Assay:

Various components of this radioreceptor assay were carefully examined in the process of designing the present version. Efforts were directed toward "optimizing" the assay with respect to conserving binding sites (for example by minimizing loss through degradation and poor retention, and minimizing dissociation). In numerous preliminary studies the following specific aspects of this assay were evaluated: (i) protocol for separation of bound from free radioligand, (ii) the design of the filtration apparatus, (iii) selection of optimal filter material, (iv) maximization of the retention of tissue bound radioligand and minimization of adsorption of radioligand to filters and non-specific

binding of radioligand to tissue, (v) conservation of bound radioactivity, (vi) evaluation of various goldfish pituitary preparations to localize binding subcellularly and minimize nonspecific binding of radioligand, and (vii) selection of optimal incubation temperature to reveal maximal numbers of binding sites and prevent loss through temperature-related degradation. Although the findings of these investigations were central to the design of the radioreceptor assay they do not constitute "original findings"; consequently these findings are summarized in Appendix II.

Whole pituitary glands (average wet weight 2 mg) were rinsed three times with excess buffer then homogenized in 5 ml of buffer with 8 strokes of a motor-driven Teflon:glass homogenizer (0.125 mm clearance). The pituitary homogenate was then diluted to the desired concentration with buffer.

The assay was conducted in 16 X 100 mm borosilicate culture tubes, and the assay mixture consisted of 3 ml of buffer, 1 ml of [^{3}H]-spiperone (New England Nuclear, Boston, Massachusetts, 22-28 Ci/mmole), 1 ml of pituitary homogenate or buffer, and 1 ml of unlabelled competitor or buffer. The reaction was initiated by addition of pituitary homogenate, incubated at 0-4 ^{o}C for defined periods, and then terminated by rapid vacuum filtration ($\leq$ 3 sec) of the incubated material through Whatman GF/B filters (presoaked for 36 h in buffer), followed immediately by a rinse with 5 ml of buffer. Filters were placed into 20-ml glass scintillation vials and dried in an airstream at room temperature for 24-48 h. After the filters were dried, 10 ml of a toluene-based scintillation cocktail (toluene 700 ml:methoxyethanol 300

ml; POPOP 0.1 g, PPO 4 g) was added to each vial; the vials were capped and incubated at room temperature, in the dark, overnight until the filters were translucent. [^{3}H]-Radioactivity was counted in an LKB 1217 liquid scintillation counter at approximately 29 % efficiency.

Specific Investigations:

A. Effect of tissue content on total and specifically bound [^{3}H]-spiperone and tissue specificity of specific [^{3}H]-spiperone binding.

[^{3}H]-Spiperone (1.6×10^{-9} M) was incubated for 2 h with buffer (blank binding) or with various concentrations of pituitary homogenate in the absence (total binding) or in the presence (non-specific binding, "NSB") of 10^{-5} M domperidone (Janssen Pharmaceutica, Beerse, Belgium). Specific binding was defined as the difference in bound radioactivity between total and NSB.

In another protocol goldfish were anaesthetized and blood sampled by puncture of caudal vessels as described in Omeljaniuk et al. (1987); fish were killed and various tissues were removed, pooled and prepared for the radioreceptor assay. Whole blood was centrifuged at approximately 10000 g X 30 min at 4°C and an aliquot of the pelleted cells was weighed, homogenized and diluted to 2 mg wet weight per ml of buffer; similarly, brain, testes and liver were prepared and all preparations were used as 1 ml per incubation tube. Tissues were incubated with [^{3}H]-spiperone as above except for 30 min.

B. Nature of [^{3}H]-radioactivity in the radioreceptor assay.

[^{3}H]-Spiperone (1.8×10^{-9} M) was incubated for 2 h with pituitary homogenate in the absence or presence of 10^{-5} M domperidone. After 2 h of incubation, material was sampled for extraction; samples of a [^{3}H]-spiperone standard in buffer or incubated material were extracted with chloroform:methanol (9:1) under nitrogen, in darkness, overnight. Aliquots of extracted material were concentrated by evaporation under nitrogen. Extracts were chromatographically evaluated by thin layer chromatography (TLC) on silica gel G TLC plates (Fisher) in a solvent system of chloroform:methanol (9:1). [^{3}H]-Radioactivity in TLC fractions was counted as previously described.

C. Association of specifically bound [^{3}H]-spiperone to goldfish pituitary homogenate.

In 3 separate experiments [^{3}H]-spiperone ($0.13\text{-}1.3 \times 10^{-9}$ M) was incubated with pituitary homogenate in the absence or in the presence of 1×10^{-5} M domperidone for various periods of time.

D. Dissociation of specifically bound [^{3}H]-spiperone from goldfish pituitary homogenate.

In 2 separate experiments [^{3}H]-spiperone (0.12×10^{-9} M) was incubated with pituitary homogenate for 30 min in parallel groups of tubes before one group of tubes received 20 ul of a domperidone solution (final concentration 10^{-5} M). Thereafter, the reaction was allowed to proceed for various durations.

E. Saturation analysis of the goldfish pituitary [^{3}H]-spiperone binding sites.

In 3 separate experiments goldfish pituitary homogenate was incubated for 30 min with increasing concentrations of combinations of unlabelled spiperone (Janssen Pharmaceutica) and [^{3}H]-spiperone, in the absence or presence of 10^{-5} M domperidone.

F. Displacement analysis of the goldfish pituitary [^{3}H]-spiperone binding sites.

In 5 separate experiments goldfish pituitary homogenate was incubated for 30 min with [^{3}H]-spiperone (0.1-0.4 X 10^{-9} M) in the absence or presence of various concentrations of domperidone.

G. Effect of dopamine antagonists and dopamine agonists on [^{3}H]-spiperone binding to goldfish pituitary homogenate.

Goldfish pituitary homogenate was incubated for 30 min with [^{3}H]-spiperone (0.1-0.4 X 10^{-9} M) in the absence or presence of various concentrations of dopamine antagonists (domperidone, spiperone, pimozide (Janssen) or (+)- or (-)-sulpiride (Ravizza s.p.a., Milan, Italy), metoclopramide (Sigma, St. Louis, MO, USA)) or dopamine-agonists ((+)- or (-)-apomorphine (Research Biochemicals Inc., Natick, MD, USA), (+/-)-apomorphine (Sigma), pergolide (a generous gift from Dr. Glen Baker, Neurochemical Research Unit, Dept. Psychiatry, University of Alberta, Edmonton, Canada), lisuride (Schering AG, Berlin, W.Germany), bromocryptine (Sigma)).

H. Displacement analysis of the [^{3}H]-spiperone binding site in the pars distalis (PD) or neurointermediate lobe (NIL) of the goldfish pituitary.

The PD was surgically separated from the NIL *in vitro* and lobes were pooled and prepared for assay similar to whole pituitaries. Homogenates of the PD or NIL were incubated for 30 min with [^{3}H]-spiperone (0.1-0.2 X 10^{-9} M) in the absence or presence of various concentrations of domperidone.

Determination of protein content.

Determination of pituitary protein content was made by the Bradford method (Bradford, 1976) using Bio-Rad dye reagent (Bio-Rad Laboratories, Richmond, California) and bovine serum albumin (Sigma, St. Louis, Missouri) as a protein standard.

Data analysis.

Where indicated, a computerized non-linear least squares curve fitting program (LIGAND) was used for Scatchard analysis and determination of best fit lines (Munson & Rodbard, 1980). A computerized four-parameter logistic curve fitting program (ALLFIT) was used for analysis of curves and determination of ED_{50} values (DeLean, Munson & Rodbard, 1978).

RESULTS

A. Effect of tissue content and type on total and specifically bound [^{3}H]-spiperone.

Total and specific [^{3}H]-spiperone binding to goldfish whole pituitary homogenate increased directly with tissue content (Figure IV-1A). Specific binding approached maximum values of 6-7 % of total radioactivity at 2 or more pituitary-equivalents per tube (approximately 22 ug protein/5 ml). In contrast, total binding using up to 4 pituitary equivalents per tube represented approximately 20 % of total radioactivity and did not appear to approach maximal levels. At a tissue content of 1-pituitary-equivalent/tube total and specifically bound [^{3}H]-spiperone represented approximately 10 and 5 % of total radioactivity, respectively.

Absolute amounts of specifically bound [^{3}H]-spiperone varied with the nature of the tissue (Figure IV-1B). The largest amount of specific [^{3}H]-spiperone binding, adjusted for tissue mass, was observed in pituitary homogenate; less binding was observed in preparations of brain, testes and liver whereas there was minimal binding to a preparation of blood cells.

B. Nature of [^{3}H]-radioactivity in the radioreceptor assay.

The TLC chromatographic profile of [^{3}H]-spiperone, diluted in assay buffer, appeared as a single major peak with an Rf value of approximately 0.42-0.47 (Figure IV-2). Organic extracts of aliquots of pituitary homogenate incubated with [^{3}H]-spiperone in the absence or

presence of 10^{-5} M domperidone had chromatographic profiles similar to the standards.

C. Association of specifically bound [^{3}H]-spiperone to goldfish pituitary homogenate.

Specific binding of [^{3}H]-spiperone to goldfish whole pituitary homogenate increased rapidly after initiation of incubation (Figure IV-3). Maximal levels of specific binding occurred at 30 min and were about constant through to 8 h.

D. Dissociation of specifically bound [^{3}H]-spiperone from goldfish pituitary homogenate.

[^{3}H]-Spiperone, specifically bound under equilibrium conditions, dissociated rapidly upon addition of excess domperidone (Figure IV-4). The dissociation kinetics of specifically bound [^{3}H]-spiperone exhibited a single component with an estimated half-life ($t_{1/2}$) of 9.2 min, and an estimated dissociation rate constant (k_{-1}) of 7.56 X 10^{-2} min^{-1} (Bennet & Yamamura, 1985).

E. Saturation analysis of the goldfish pituitary [^{3}H]-spiperone binding site.

Spiperone bound to goldfish pituitary homogenate in a saturable fashion (Figure IV-5). ALLFIT analysis of the data indicated the presence of a single class of binding site with an estimated dissociation constant (Kd) of 7.39 $\pm$ 1.23 X 10^{-6} M and an estimated capacity of 3.03 $\pm$ 0.261 X 10^{-9} moles/pituitary (31.56 $\pm$ 2.72 X 10^{-9} moles/mg protein). Simple Scatchard analysis (Scatchard, 1949) of the

data (Figure IV-5 inset) estimated the Kd as 2.1×10^{-5} M and the capacity as 5.75×10^{-9} moles/pituitary.

F. Displacement analysis of the goldfish pituitary [^{3}H]-spiperone binding site.

Domperidone displaced [^{3}H]-spiperone from goldfish pituitary homogenate in a dose-related fashion (Figure IV-6). LIGAND analysis of the data indicated the presence of a single class of binding sites with a Kd of $2.94 \pm 0.54 \times 10^{-6}$ M and a capacity of $2.09 \pm 0.23 \times 10^{-9}$ moles/pituitary ($19.47 \pm 3.12 \times 10^{-9}$ moles/mg protein). Computerized Scatchard analysis (Figure IV-6 inset) of the data similarly indicated a single class of binding sites.

G. Effect of dopamine antagonists and dopamine agonists on [^{3}H]-spiperone binding to goldfish pituitary homogenate.

[^{3}H]-Spiperone was displaced by spiperone and domperidone in a similar fashion and to equivalent degrees (Figure IV-7A). In contrast, (+)- and (-)-sulpiride shared a common potency to displace [^{3}H]-spiperone but whose apparent minimum effective doses (approximately 10^{-5} M) was approximately 100 fold greater than that of spiperone or domperidone (approximately 10^{-7} M). Pimozide had a potency to inhibit [^{3}H]-spiperone binding similar to that of domperidone (Figure IV-7B) whereas metoclopramide was less potent and had an approximate ED_{50} of $10^{-4.7}$ M.

Data from 4 independent experiments indicate that apomorphine, either separated optical isomers or a racemic mixture, inhibited [^{3}H]-spiperone binding by 90 % between 10^{-7} and 10^{-4} M (Figure IV-7C).

Pergolide and lisuride had similar potencies for inhibition of [^{3}H]-spiperone binding (ED_{50} approximately $10^{-5.3}$ M; bromocryptine inhibited [^{3}H]-spiperone binding by only 30 % between 10^{-6} and 10^{-4} M.

H. Displacement analysis of the [^{3}H]-spiperone binding site in the PD or NIL of the goldfish pituitary.

Domperidone displaced [^{3}H]-spiperone from homogenates of the PD and NIL of the goldfish pituitary gland in dose-related fashions (Figure IV-8). LIGAND analysis of these data indicated the existence of a single class of binding site in the PD and NIL. The binding sites in both lobes shared similar Kd's of $3.73 \pm 0.248 \times 10^{-6}$ M and $4.1 \pm 1.21 \times 10^{-6}$ M for the PD and NIL, respectively. In contrast, the capacity of the PD ($38.89 \pm 2.07 \times 10^{-9}$ moles/mg protein) was significantly ($p<0.05$) smaller than that of the NIL ($109.45 \pm 25.33 \times 10^{-9}$ moles/mg protein).

DISCUSSION

The existence of a teleost pituitary dopamine receptor has been suggested on the basis that dopamine inhibits the release of several pituitary hormones such as prolactin (Olivereau, 1975), and o -MSH (Olivereau, 1978; Olivereau et al., 1987). In particular, recent evidence strongly indicates that dopamine is a potent gonadotropin release inhibiting factor (GRIF) in goldfish and other teleosts (reviewed by Peter et al., 1986). Furthermore, the presence of dopamine/neuroleptic receptors in the pituitary is indicated by the finding that the goldfish pituitary preferentially concentrated intraperitoneally injected [^{3}H]-domperidone, relative to whole blood, the gonads, and the brain (this thesis, Chapter II).

The teleosts lack a functional hypothalamo-hypophyseal blood portal system, but both the PD and NIL are directly innervated by neurosecretory fibers (Ball, 1981). In the goldfish, gonadotrophs in particular are directly innervated by neurons reacting to antisera against GnRH (Kah, Breton, Dulka et al., 1986a) as well as by neurons reacting to antisera against dopamine (Kah, Dubourg, Ontenient et al., 1986b). In addition, a recent in vivo investigation using dopamine receptor-specific antagonists suggests that in goldfish dopamine inhibits GtH secretion by stimulating a pituitary dopamine/neuroleptic (D2-like) receptor (this thesis, Chapter II).

In the present investigation, I have used [^{3}H]-spiperone to characterize the binding parameters of a spiperone/neuroleptic binding site. Spiperone is a dopamine:D2-selective receptor antagonist that has

been used widely to identify brain (reviewed by Seeman, 1981) and pituitary (reviewed by Cronin, 1982) dopamine receptors. The findings on the binding of [^{3}H]-spiperone to goldfish pituitary homogenate satisfy most of the criteria related to ligand:receptor interactions as outlined by Cronin (1982), Laduron (1984), and Leysen (1984); binding was tissue-specific and the magnitude of binding was found to be dependent on tissue (protein) content and specific binding to be heat-labile (data not shown); association was rapid and binding was reversible (dissociable) by addition of excess competing ligand; the association rate was temperature-dependent (data not shown); although binding was of relatively low affinity it was saturable, displaceable, and specifically inhibited by drugs from different chemical classes; finally, density of binding sites, relative to protein content, differed significantly between regions of the goldfish pituitary. The data indicate that under the conditions of this radioreceptor assay [^{3}H]-spiperone is identifying a single type of dopamine/neuroleptic receptor.

Based on the currently accepted classification of dopamine receptors, D1 and D2, (Leff & Creese, 1984) the goldfish pituitary spiperone/neuroleptic binding site more closely resembles the dopamine:D2 receptor. Investigations of pituitary dopamine receptors indicate that the mammalian pituitary contains only the dopamine:D2 receptor subtype; [^{3}H]-spiperone has been used to label a mammalian pituitary dopamine:D2 receptor (K_d approximately 0.13 X 10^{-9} M) (George, Watanabe & Seeman, 1985). The pituitary dopamine:D2 receptor is most commonly associated with prolactin-secreting cells in the pars distalis (Cronin, 1982) and with α-MSH-secreting cells in the pars intermedia (Cote et al., 1982). The dopamine:D2 receptor, in contrast to the

dopamine:D1 receptor, is sensitive to substituted benzamides and can discriminate between the active and inactive stereoisomers (-)- and (+)-sulpiride, respectively (Grigoriadis & Seeman, 1984). As well, dopamine:D2 receptors exist in high- and low-affinity states; D2-high is readily convertable to D2-low *in vitro* (George *et al.*, 1985). Our present study using [^{3}H]-spiperone indicates that goldfish pituitary dopamine receptors consist of a single class of low affinity (Kd approximately 3-7 X 10^{-6} M) binding sites which do not appear to be stereoselective for the isomers of sulpiride or apomorphine. Both saturation and displacement protocols yielded comparable values for affinity (Kd estimated from saturation analysis was 7.4 $\pm$ 1.2 X 10^{-6} M vs 2.9 $\pm$ X 10^{-6} M for the displacement analysis estimate of Kd) and capacity (31.6 $\pm$ 2.7 X 10^{-9} moles/mg protein determined from saturation analysis vs 19.5 $\pm$ 3.1 X 10^{-9} moles/mg protein determined from displacement analysis). Previously, on the basis of preliminary experiments, we reported the saturable binding of [^{3}H]-spiperone to goldfish pituitary homogenate with an estimated Kd of 1.95 X 10^{-9} M (Peter *et al.*, 1986). In spite of subsequent repeated trials, we have not been able to confirm the existence of this high affinity binding site.

Domperidone has been shown to be a potent and specific dopamine-receptor antagonist (Sowers, Sharp & McCallum, 1982), and [^{3}H]-domperidone has been used in *in vitro* radioreceptor assays to identify dopamine:D2 receptors in striatal membrane preparations (Martres, Baudry & Schwartz, 1978) and on intact pituitary cells in culture (Foord, Peters, Dieguez, Scanlon & Hall, 1983). The micromolar potency of domperidone for the displacement of [^{3}H]-spiperone from

goldfish pituitary homogenate is consistent with our findings of the micromolar potency of domperidone to reverse dopamine inhibition of GnRH-stimulated GtH secretion in vitro (this thesis, Chapter III). By way of comparison, recent studies have shown that in the goldfish there are two classes of pituitary binding sites for GnRH with Kd's in the nanomolar and sub-nanomolar range (Habibi, Peter, Sokolowska et al., 1987). Comparison of the molar effectiveness of domperidone in vitro (this thesis, Chapter III) versus the effective dosages of GnRH in vitro (MacKenzie, Gould, Peter et al., 1984) indicates that GnRH's are effective at nanomolar concentrations, whereas domperidone is effective at micromolar concentrations. These findings imply that, in the goldfish pituitary, GnRH's interact with high affinity (Kd$\leq 10^{-9}$ M) GnRH receptors whereas dopamine antagonists interact with low affinity (Kd approximately 10^{-6} M) dopamine/neuroleptic receptors.

The present study shows that the binding of [^{3}H]-spiperone to goldfish pituitary homogenate was inhibited, but not stereospecifically, by the substituted benzamides (+)- and (-)-sulpiride. This finding is not consistent with the properties of the classical high-affinity dopamine:D2 receptor (Leff & Creese, 1984). However, in keeping with the low affinity nature of this binding site, the lack of demonstrable stereospecificity is not unexpected (Hoyer, 1986). We have previously demonstrated that (-)-sulpiride caused a small dose-related increase in circulating GtH levels in goldfish, whereas (+)-sulpiride was ineffective; domperidone was far more potent than (-)-sulpiride in this regard (this thesis, Chapter II). Data from in vitro studies suggest weak to nonexistent stereoselectivity for inhibition of GtH and α-MSH release by isomers of apomorphine (this thesis, Chapter III); results

from preliminary *in vitro* studies also indicate little to no stereoselectivity for antagonism of dopamine action by stereoisomers of sulpiride. Endogenous catecholamines are highly labile and thus are poor ligands to use for evaluation of receptor binding site parameters in radioreceptor assays that do not contain protective substances such as ascorbic acid; as well, endogenous catecholamines do not specifically identify catecholaminergic receptor subtypes. As dopamine cannot resolve dopamine receptor subtypes, [^{3}H]-spiperone, the most widely accepted dopamine receptor probe, has been used in this study to examine the goldfish dopamine/neuroleptic receptor. In mammals, [^{3}H]-spiperone has been used to identify dopamine:D2 receptors with the limitation that at high concentrations (uM) spiperone may interact with serotonin binding sites, for example in brain tissue (Seeman, 1981); to minimize this possibility, [^{3}H]-spiperone was used in low concentrations (nM) in displacement experiments in this study. To date, the bulk of evidence indicates that domperidone is a specific dopamine:D2 receptor antagonist; there is no evidence to indicate that domperidone interacts with serotonin binding sites. The results of this study suggest that [^{3}H]-spiperone and domperidone identify a goldfish pituitary dopamine/neuroleptic receptor. Ketanserin in mammals is specific $5HT_2$ receptor antagonist which shares some structural similarities with domperidone. Preliminary studies using goldfish pituitary tissue indicate that ketanserin significantly inhibited [^{3}H]-spiperone binding; however, the significance of this anomalous finding is not clear as ketanserin binding does not appear to correlate with the known actions of ketanserin on GtH secretion *in vivo* (Somozoa & Peter, unpublished results). These data suggest that although [^{3}H]-spiperone identifies a

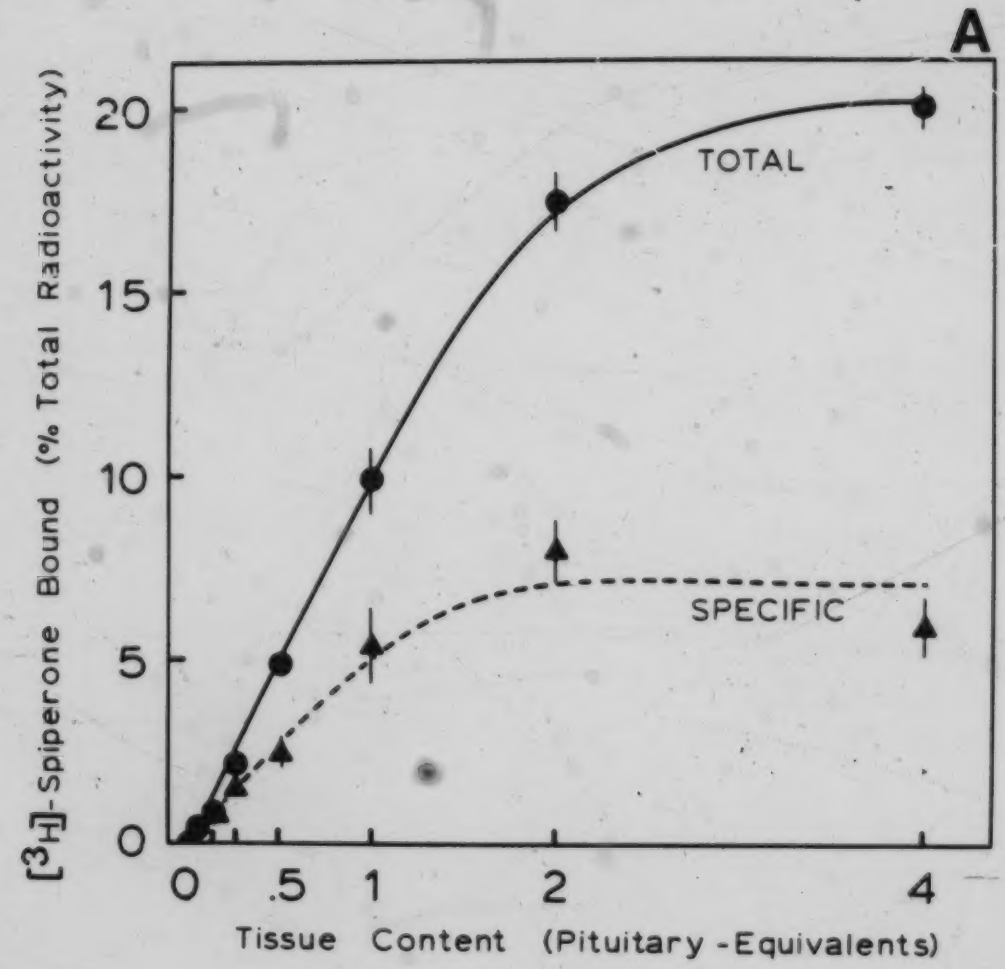
dopamine/neuroleptic receptor in the goldfish pituitary, there are aspects of binding specificity which may not be strictly in accord with the classical definition of the dopamine:D2 receptor in mammals.

Our results indicate that the NIL of the goldfish pituitary contained a significantly greater number of dopamine/neuroleptic receptors than the PD; this finding subsequently has been confirmed (this thesis, Chapter V). The goldfish NIL is richly innervated by dopamine-immunoreactive neurons (Kah et al., 1986a), and there is substantial evidence for the inhibition of α-MSH secretion by dopamine in teleosts (Olivereau, 1978; Olivereau et al., 1987; this thesis, Chapter III), amphibians (Tonon, 1984), and mammals (Cote et al., 1982).

In summary, this study provides evidence for the existence of a goldfish pituitary [^{3}H]-spiperone/neuroleptic binding site that has the binding characteristics of a receptor. This is a low affinity, high capacity binding site whose pharmacological characteristics, tissue distribution, and apparent functional relationship to the dopamine antagonist domperidone resemble those of a dopamine:D2 receptor. This is the first demonstration of the existence and binding characteristics of a dopamine:D2-like receptor in the pituitary of a non-mammalian vertebrate.

Figure IV-1A: Binding of [^{3}H]-spiperone (% of total radioactivity) to various concentrations of goldfish pituitary homogenate; total (circles) and specific (triangles) binding; non-specific binding=[^{3}H]-spiperone bound in the presence of 10^{-5} M domperidone. Values are means +/- S.E.M. (n=5-6).

Figure IV-1B: [^{3}H]-Spiperone (cpm/mg) specifically bound to various goldfish tissue preparations using approximately 2 mg (wet weight) of tissue equivalent per assay tube; 1-pituitary equivalent is approximately 2 mg (wet weight). Values are means $\pm$ S.E.M. (n=6).


A
20
15
10
5
0
TOTAL
SPECIFIC
[3H]-Spiperone Bound (% Total Radioactivity)
0
.5
1
2
4
Tissue Content (Pituitary-Equivalents)

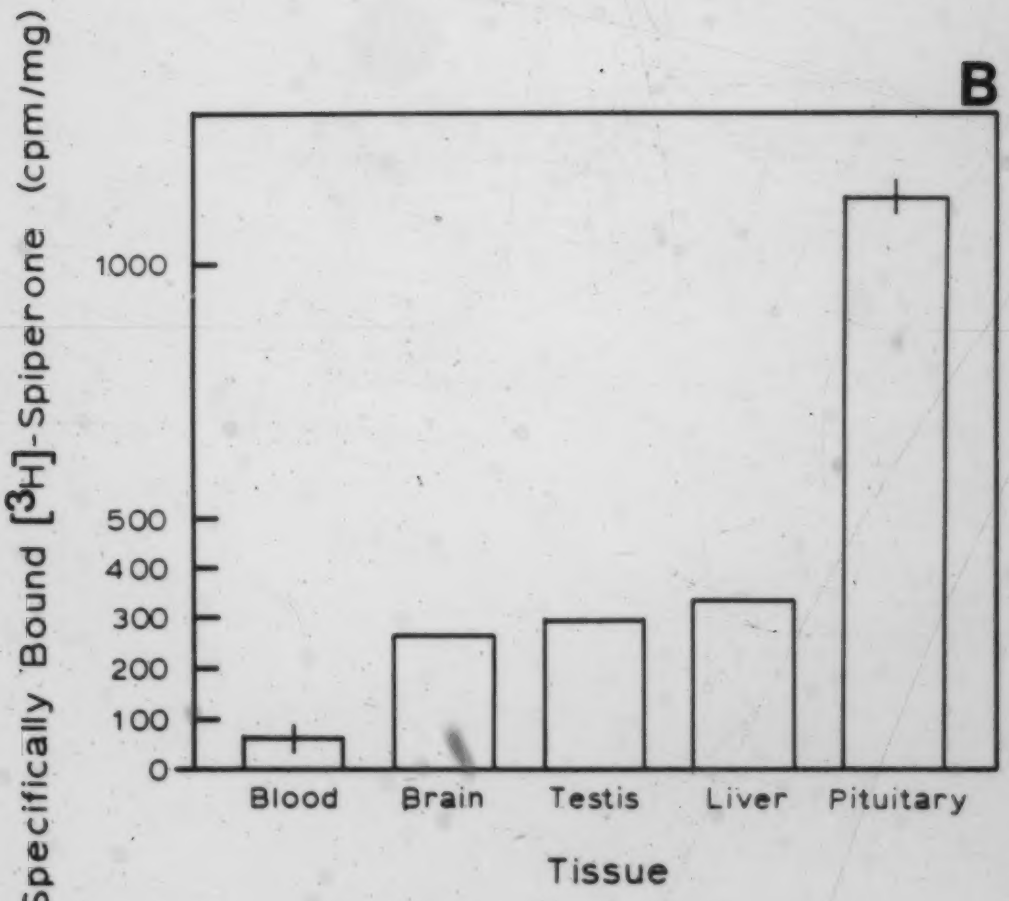

B
Specifically Bound [3H]-Spiperone (cpm/mg)
1000
500
400
300
200
100
0
Blood
Brain
Testis
Liver
Pituitary
Tissue

Figure IV-2: [^{3}H]-Radioactivity (cpm) in 1-cm fractions of silical gel G TLC chromatograms of (i) [^{3}H]-spiperone standard in assay buffer; organic extracts of incubated material without (ii) or with (iii) 10^{-5} M domperidone. Arrows indicate solvent front.

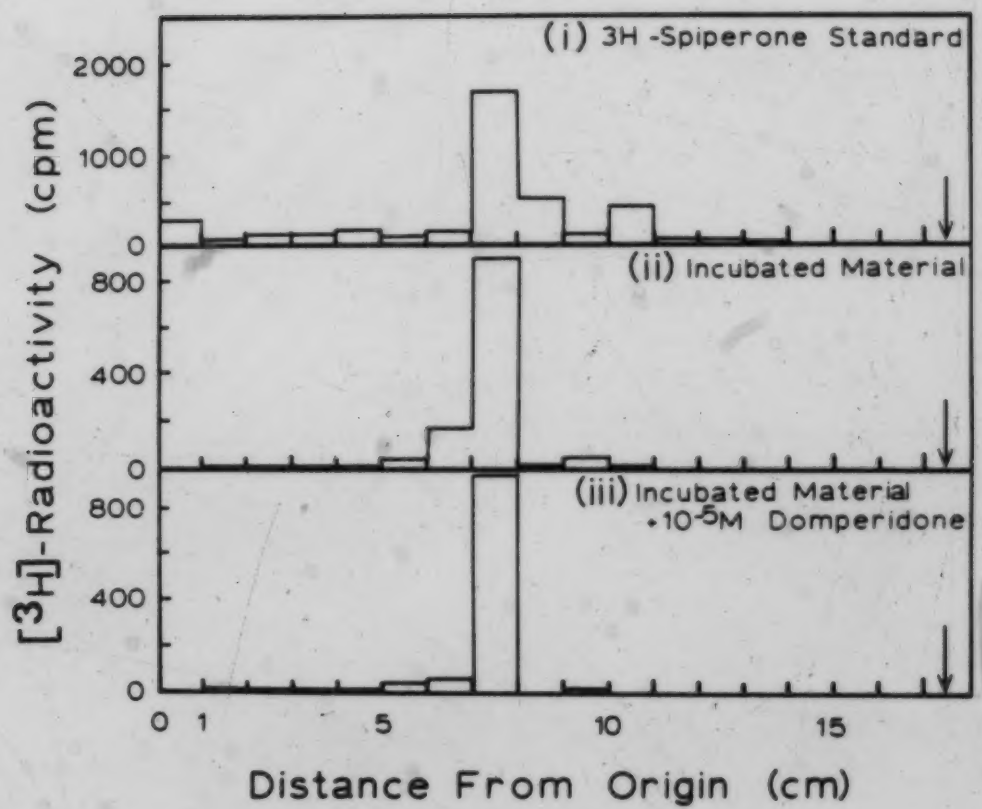

(i) 3H -Spiperone Standard
(ii) Incubated Material
(iii) Incubated Material
+10-5M Domperidone
[3H]-Radioactivity (cpm)
2000
1000
0
800
400
0
800
400
0
0 1 5 10 15
Distance From Origin (cm)

Figure IV-3: Association of specifically bound [^{3}H]-spiperone (% maximum specific binding) as a function of incubation time. Values are means +/- S.E.M. (n=3) from 3 independent experiments.

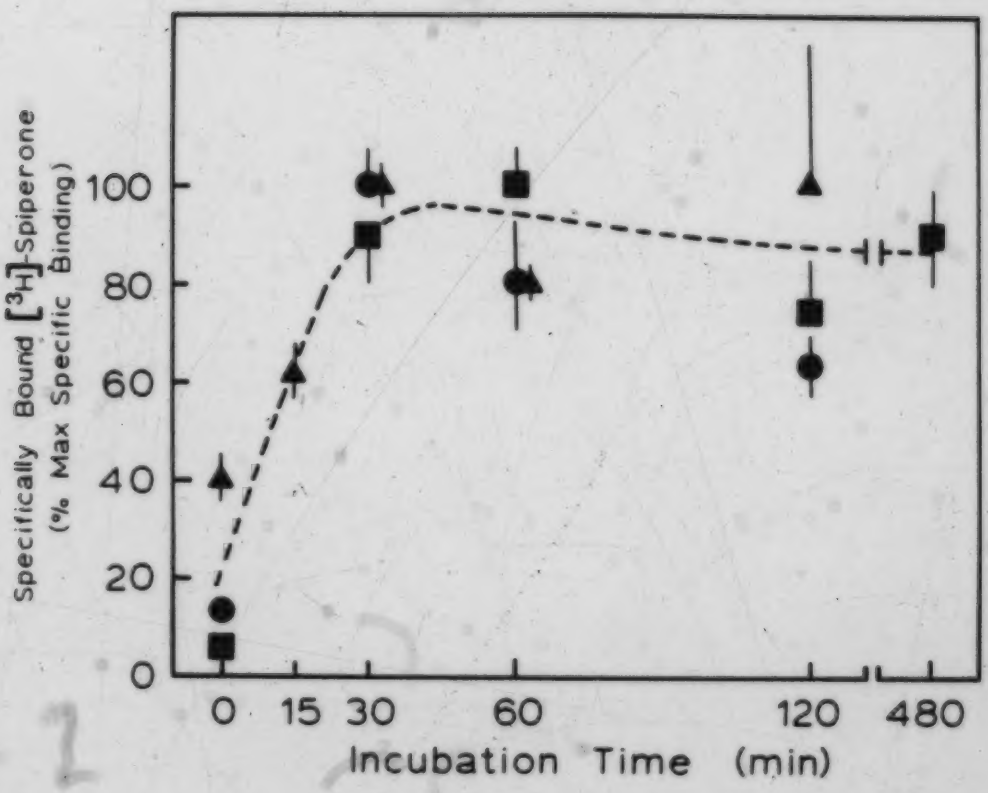

Specifically Bound [3H]-Spiperone
(% Max Specific Binding)
0
20
40
60
80
100
0
15
30
60
120
480
Incubation Time (min)

Figure IV-4: Dissociation of [^{3}H]-spiperone (% maximum specific binding) bound at equilibrium to goldfish pituitary homogenate after addition of domperidone (final concentration 10^{-5} M). Values are means +/- S.E.M. (n=3) from 2 independent experiments. Mathematically estimated half-life ($t_{1/2}$), and dissociation rate constant (k_{-1}) were 9.2 min and 7.56 X 10^{-2} min^{-1}, respectively (Bennet & Yamamura, 1985).

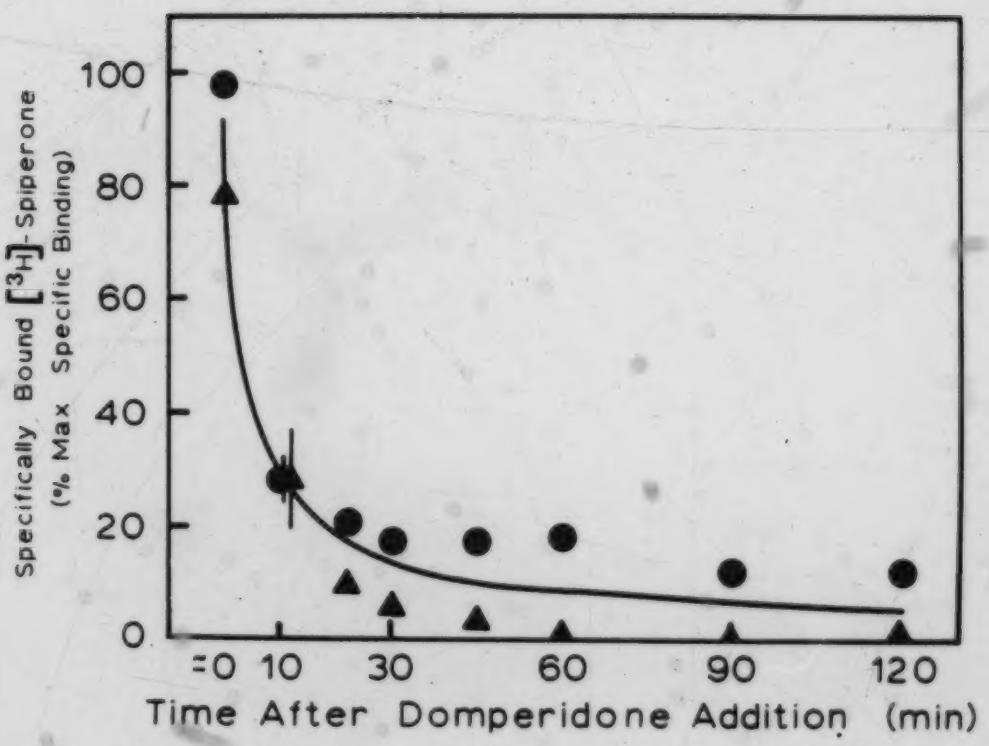

Specifically Bound [3H]-Spiperone
(% Max Specific Binding)
100
80
60
40
20
0
=0
10
30
60
90
120
Time After Domperidone Addition (min)

Figure IV-5: Saturation analysis of the goldfish pituitary [^{3}H]-spiperone binding site. Specifically bound spiperone (X 10^{-9} moles) as a function of ambient spiperone (M). Values are means +/- S.E.M. (n=3) from 3 independent experiments. For purposes of clarity not all points at $\leq$ 1 X 10^{-6} M are shown. Inset: simple Scatchard analysis of data. Estimates of Kd were 7.39 $\pm$ 1.23 X 10^{-6} M (ALLFIT) vs 2.1 X 10^{-5} M (simple Scatchard); capacity was 31.56 $\pm$ 2.72 X 10^{-9} moles/mg protein (ALLFIT).

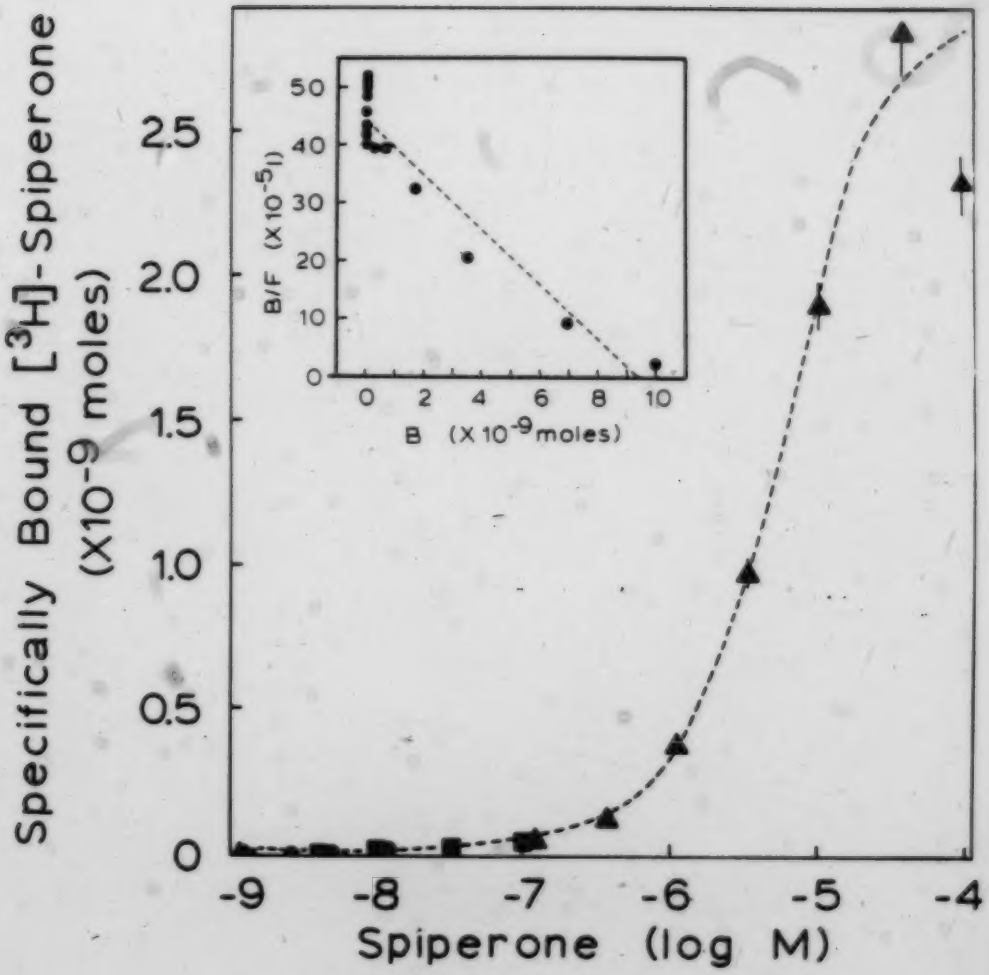

Specifically Bound [3H]-Spiperone
(X10-9 moles)
2.5
2.0
1.5
1.0
0.5
0
-9
-8
-7
-6
-5
-4
Spiperone (log M)
B/F (X10-5l)
50
40
30
20
10
0
0
2
4
6
8
10
B (X 10-9moles)

Figure IV-6: Displacement analysis of the goldfish pituitary [^{3}H]-spiperone binding site. [^{3}H]-Spiperone bound (% B_o; binding in the absence of competitor) to goldfish pituitary homogenate as a function of ambient domperidone concentration (M). Values are means +/- S.E.M. (n=3) from 5 independent experiments. Inset: LIGAND based Scatchard analysis of the same data; overall means of B/F at a given domperidone concentration. Estimates of binding parameters were Kd=2.94 $\pm$ 0.54 X 10^{-6} M and capacity=19.47 $\pm$ 3.12 X 10^{-9} moles/mg protein.

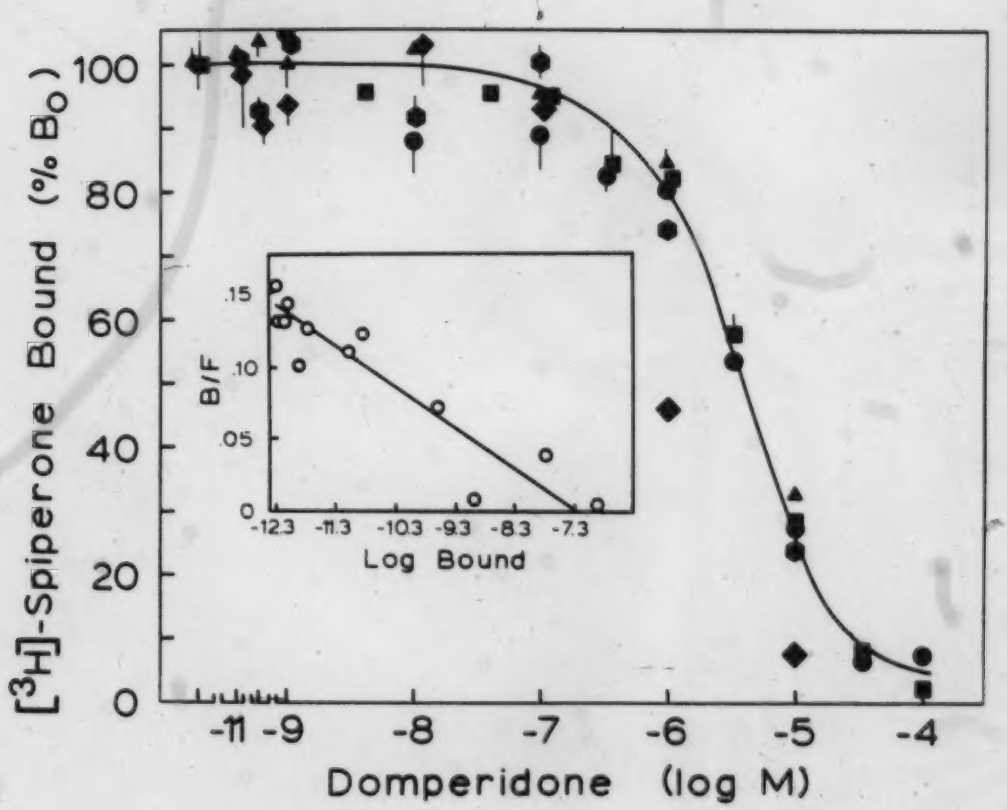
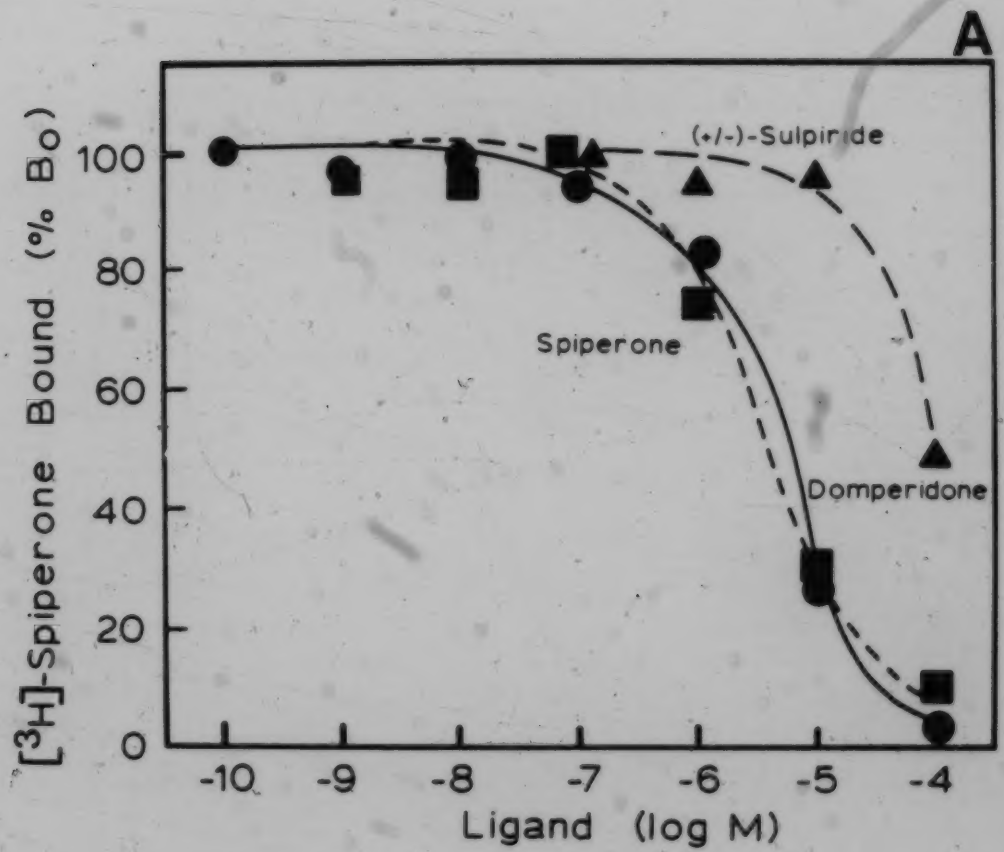

[3H]-Spiperone Bound (% Bo)
100
80
60
40
20
0
-11 -9 -8 -7 -6 -5 -4
Domperidone (log M)
B/F
.15
.10
.05
0
-12.3 -11.3 -10.3 -9.3 -8.3 -7.3
Log Bound

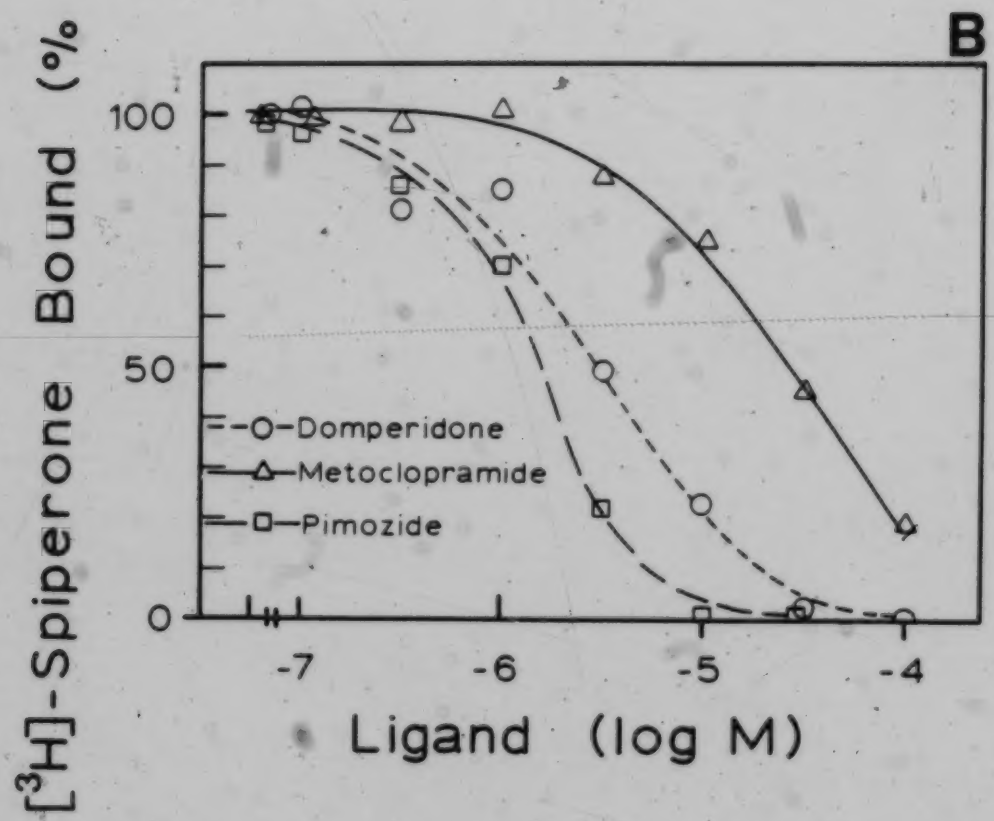
Figure IV-7: Effect of dopamine antagonists and dopamine agonists on binding of [^{3}H]-spiperone to goldfish pituitary homogenate.

A. [^{3}H]-Spiperone bound (% B_o; binding in the absence of competitor) to goldfish pituitary homogenate as a function of ambient competitor concentration (M): spiperone (squares), domperidone (circles), (+/-)-sulpiride (triangles). Values are means +/- S.E.M. (n=3) from 4 independent experiments.

B. Comparison of domperidone, metoclopramide, and pimozide inhibition of [^{3}H]-spiperone binding to goldfish pituitary homogenate. Values are means $\pm$ S.E.M. (n=3) from a single experiment.

C. Comparison of the effects of the dopamine agonists pergolide, lisuride, bromocryptine, and preparations of isomers of apomorphine on inhibition of [^{3}H]-spiperone binding to goldfish pituitary homogenate. Values are means $\pm$ S.E.M. (n=3) from 2 independent experiments.


A
(+/-)-Sulpiride
Spiperone
Domperidone
[3H]-Spiperone Bound (% Bo)
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
-4
Ligand (log M)


B
100
50
0
-7
-6
-5
-4
-O-Domperidone
-Δ-Metoclopramide
-□-Pimozide
[3H]-Spiperone Bound (% B0)
Ligand (log M)

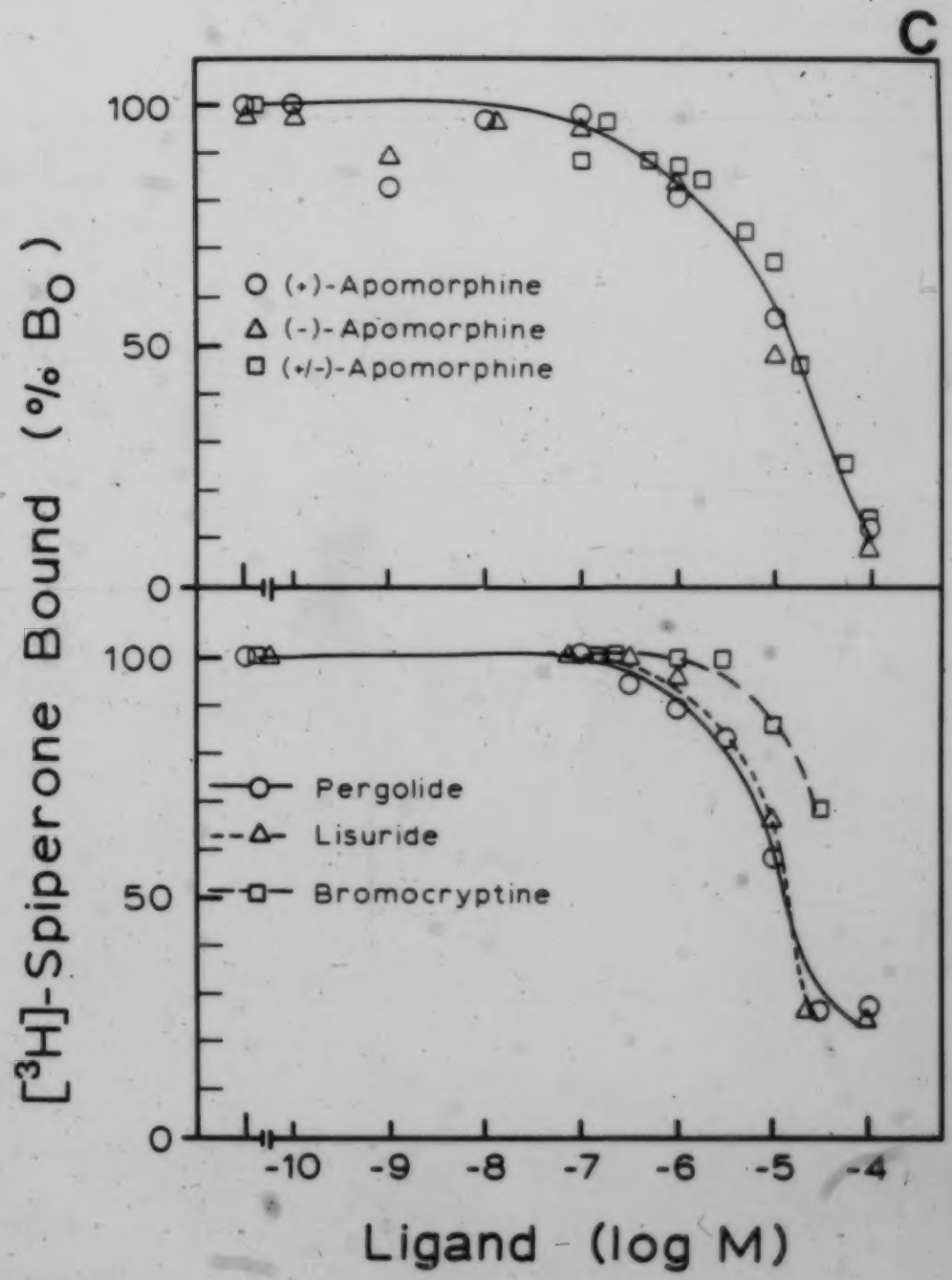
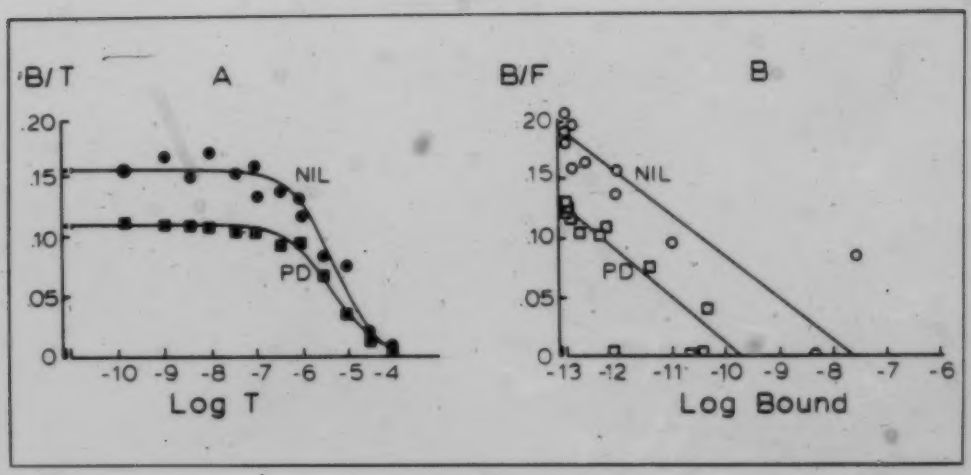

C
100
50
0
(+)-Apomorphine
(-)-Apomorphine
(+/-)-Apomorphine
100
50
0
Pergolide
Lisuride
Bromocryptine
[3H]-Spiperone Bound (% Bo)
-10
-9
-8
-7
-6
-5
-4
Ligand (log M)

Figure IV-8: Displacement analysis of the [^{3}H]-spiperone binding site in the PD and NIL of the goldfish. A. [^{3}H]-Spiperone bound (B/T; binding in the absence of competitor expressed as the proportion of total radioactivity) to goldfish PD and NIL homogenate as a function of ambient domperidone concentration (M). Values are means +/- S.E.M. (n=3) from 2 independent experiments. Curves are based on LIGAND analysis of the data. PD (squares), NIL (circles). B. LIGAND based Scatchard analysis of the same data. Estimates of binding parameters were Kd=3.73 $\pm$ 0.248 X 10^{-6} M for PD vs Kd=4.1 $\pm$ 1.21 X 10^{-6} M for NIL; capacity estimates were 38.89 $\pm$ 2.07 X 10^{-9} moles/mg protein for PD vs 109.45 $\pm$ 25.33 X 10^{-9} moles/mg protein for NIL. PD (open squares), NIL (open circles).


B/T
A
.20
.15
.10
.05
0
NIL
PD
-10 -9 -8 -7 -6 -5 -4
Log T
B/F
B
20
.15
.10
.05
0
NIL
PD
-13 -12 -11 -10 -9 -8 -7 -6
Log Bound

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V. Alterations in pituitary GnRH and dopamine receptors associated with the seasonal variation and regulation of gonadotropin (GtH) release in the goldfish (*Carassius auratus*).

INTRODUCTION

In goldfish, and other teleost fishes, GnRH and dopamine act directly on the pituitary to regulate the release of GtH; GnRH stimulates GtH release whereas dopamine modulates the spontaneous release of GtH and inhibits the action of GnRH (for review see Peter, Chang, Nahorniak *et al.*, 1986). We have previously demonstrated that domperidone, a specific dopamine receptor antagonist, injected i.p. into goldfish increased serum concentrations of GtH in a dose-related manner (this thesis, Chapter II); domperidone and [D-Arg^{6}, Trp^{7}, Leu^{8}, Pro^{9}-NEt]-LHRH (sGnRH-A) exerted synergistic actions in terms of the serum GtH levels studied *in vivo*. Domperidone is a suitable dopamine antagonist for investigation of regulatory actions at the pituitary level, since it does not enter the brain but acts directly on the pituitary (this thesis, Chapter II).

In untreated male and female goldfishes, serum concentrations of GtH vary over the annual reproductive cycle; maximum GtH levels occur during advanced stages of season gonadal recrudescence (Kobayashi, Aida & Hanyu, 1986). As well, previous evidence indicates that maximal responsiveness of the pituitary to [D-Ala^{6}, Pro^{9}-NEt]LHRH (LHRH-A) and pimozide, a dopamine antagonist, occurs annually, during advanced stages of ovarian recrudescence (Sokolowska, Peter, Nahorniak *et al.*, 1985).

In mammals, domperidone specifically binds to a single class of dopamine receptors (D2 subtype) (Baudry, Martres & Schwartz, 1979), resulting in increased release of prolactin and thyrotropin by antagonizing dopamine action (Massara, Cammani, Amoroso *et al.*, 1981;

Sowers, Sharp & McCallum, 1982). In view of previous findings concerning the inhibitory action of dopamine on GnRH-induced GtH release in goldfish, we carried out studies to investigate the effects of an agonist analogue of teleost GnRH on GnRH and dopamine/neuroleptic receptors, and of domperidone, a dopamine receptor antagonist, on GnRH receptors. Also, domperidone was used in the goldfish in vivo to examine the role of dopamine in seasonal changes in regulation of GtH release and to investigate the integration of dopamine and GnRH actions on regulation of GtH release.

MATERIALS AND METHODS

Experimental animals:

Male and female common or comet variety goldfish (Carassius auratus; approximately 26 g, 10 - 12.5 cm) were obtained from Grassyforks Fisheries Co., Martinsville, IN, U.S.A. or Ozark Fisheries, Stoutland, MI, U.S.A. Fish were maintained for several weeks in flow-through aquaria at controlled (± 1 °C) ambient temperatures (7 to 19 °C, annual range) with simulated natural photoperiod (7 to 17 h photophase, annual range) (Edmonton, Canada), and were fed twice daily to excess with commercial trout food. The day before an experiment, fish were anaesthetized in tricaine methanesulphonate (0.5 g/l), weighed and identified by an opercular tag (No. 1005 Size 1 monel; National Band and Tag Co., Newport, KY, U.S.A.). In all cases, animals were anaesthetized before any handling.

Specific investigations:

A. Seasonal variability of the serum GtH response to domperidone.

At various times over a 12 month period male and female goldfish were maintained under a simulated natural temperature and photoperiod. Goldfish were segregated into groups then injected i.p. with vehicle (dimethyl sulfoxide (DMSO):propylene glycol; 1:2, v/v) or a given dose of domperidone (Janssen Pharmaceutica, Beerse, Belgium). Domperidone was dissolved in DMSO (Sigma, St. Louis, MO, U.S.A.) then diluted to final concentration for injection with propylene glycol (at a rate of 1 ml/kg body weight); fish were injected with a 25 guage (g), 16 mm needle mounted on a 50 ul Hamilton glass syringe. Blood samples were collected

24 h after injection by puncture of caudal vessels using a 25 g, 16 mm needle mounted on a 1.0-ml tuberculin syringe. Collected blood was transferred to 1.5-ml polypropylene centrifuge tubes, kept on ice for several hours and centrifuged (13000 g X 10 min at 4 °C). Serum was transferred to 500-ul polypropylene centrifuge tubes, frozen on dry ice and stored at -20 °C until radioimmunoassay (RIA) for GtH (Peter, Nahorniak, Chang & Crim, 1984).

B. Effect of multiple sGnRH-A treatments on the serum GtH response to domperidone.

Goldfish of mixed sex were maintained in 2 separate groups at simulated natural conditions (7°C; 12 h photophase). Goldfish in each group were given 3 i.p. injections, 48 h apart; one group was injected with sGnRH-A (1.5 ug/injection; a gift from J. Rivier and W. Vale, The Clayton Foundation Laboratories for Peptide Biology, Salk Institute, La Jolla, CA, U.S.A.), while another group was treated wtih vehicle. sGnRH-A was dissoved in DMSO and diluted to injection volume with propylene glycol, then injected as a 20 ul bolus in a vehicle of DMSO:propylene glycol (1:2, v/v). Twenty-four hours after each injection 10 fish from each group were blood-sampled, as described above, and returned to their group. Forty-eight hours after the final treatment the fish in each treatment group were segregated into six subgroups; fish in one subgroup were given an i.p. injection of vehicle (DMSO:propylene glycol; 1:2, v/v) while fish in other subgroups were injected with a given dose of domperidone in a total volume of 1 ml/kg body weight. Fish were blood-sampled 24 h after this injection and serum samples were subsequently analyzed for GtH content by RIA.

C. Effect of repeated treatment of goldfish with sGnRH-A on the binding parameters of [^{3}H]-spiperone to goldfish pituitary homogenate (March, 1987).

Goldfish, males and females, were segregated into 2 groups and maintained at a simulated natural temperature (11 ^{o}C) and photoperiod (12 h photophase). Fish in each group were then injected with vehicle or sGnRH-A as described in B above. Forty-eight hours after the third injection all fish received an i.p. injection of vehicle (DMSO:propylene glycol, 1:2 v/v). Twenty-four hours following the final injection, fish were anaesthetized, killed and their pituitary glands harvested. The pars distalis and neurointermediate lobes were separated, prepared, and used in a radioreceptor assay in which tissue was incubated with [^{3}H]-spiperone (New England Nuclear, (NEN) Boston, MA, U.S.A.; 22.8 Ci/mmol; 0.1-0.2 nM) and various concentrations of domperidone as described in this thesis (Chapter IV).

D. GnRH receptor assay in goldfish pituitary.

GnRH receptor characteristics were investigated in the goldfish pituitary following injection with either sGnRH-A (2 injections, 3 ug/injection, 12 h apart) or domperidone (1 injection, 40 umol/kg body weight). The fish were anaesthetized and killed at 24 h following the initial treatment, pituitaries collected and GnRH-binding properties measured as described by Habibi, Peter, Sokolowska *et al*. (1987a), using [^{125}I]-sGnRH-A as labeled ligand. GnRH receptor affinity (equilibrium association constant) and capacity (number of receptors per mg of protein) for each pituitary were estimated from displacement curves by unlabeled sGnRH-A, using LIGAND analysis. The experiments involving

treatments with sGnRH-A and domperidone were carried out separately using controls which received single or two vehicle injections according to the treatment groups, and values normalized for presentation in a single table to facilitate comparisons; the control-injected groups in the two experiments were not significantly different from each other.

Determination of protein content:

Determination of pituitary protein content was made by the Bradford method (Bradford, 1976) using Bio-Rad dye reagent (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and bovine serum albumin (Sigma) as a protein standard.

Data analysis:

Data were analyzed by one-way analysis of variance and Duncan's multiple range test (Duncan, 1955); comparisons were made at the $p < 0.05$ level of significance. A computerized four-parameter logistic curve fitting program (ALLFIT) was used for analysis of curves and determination of half-maximal effective dose (ED_{50}) values (DeLean, Munson & Rodbard, 1978). Receptor binding data was evaluated by a computerized non-linear least squares curve-fitting program (LIGAND) for Scatchard analysis and determination of best-fit lines (Munson & Rodbard, 1980).

RESULTS

A. Seasonal variability of the serum GtH response to domperidone.

Serum concentrations of GtH in goldfish injected with vehicle varied on a seasonal basis; lowest serum levels of GtH were found in sexually regressed fish (4.5 ± 0.5 ng/ml; January) while greatest levels occurred in fish undergoing advanced gonadal recrudescence (45.4 ± 14.7 ng/ml; March) (Figure V-1). At all times of the year, domperidone increased serum concentrations of GtH in a dose-related manner. The magnitude of the maximum serum GtH response to domperidone increased in correlation with advancing gonadal maturation; the smallest response occurred in sexually regressed fish in January (82.2 ± 7.8 ng/ml) while the largest response occurred in fish in late stages of gonadal recrudescence in March (608 ± 64 ng/ml). The maximum serum GtH response was lower in post-spawning fish in May and July, compared with fish in March; in sexually regressed fish in September and December the maximum serum GtH response to domperidone remained relatively stable. ALLFIT analysis of the domperidone:serum GtH response relationship indicated that the half-maximal effective dose (ED_{50}) of domperidone was approximately 13.6 umoles/kg body weight; the ED_{50} did not vary significantly over the course of the reproductive cycle. There was no apparent correlation between seasonal changes in ambient temperature or photoperiod with serum concentrations of GtH in fish treated with vehicle or in the serum GtH response to domperidone (Figure V-2).

B. Effect of multiple sGnRH-A treatments on serum concentrations of GtH and on the serum GtH response to domperidone.

Injection of sGnRH-A (i.p.) increased the serum concentraton of GtH in goldfish (Figure V-3). The GtH response to sGnRH-A increased progressively with each sGnRH-A treatment; serum concentrations of GtH remained significantly elevated as long as 72 h after the final injection of sGnRH-A compared with vehicle-injected controls (Figure V-4). Repeated injection of vehicle had no significant effect of serum concentrations of GtH (Figure V-3).

Domperidone, injected i.p., significantly increased serum concentrations of GtH, in a dose-related manner, in both vehicle- and sGnRH-A-treated goldfish (Figure V-3). sGnRH-A treatment significantly increased the magnitude of the serum GtH response to domperidone compared with vehicle-injected fish (an almost 6-fold difference); the ED_{50} for domperidone action in vehicle-treated fish of 8.5 umoles/kg body weight was reduced, but not signifcantly, by sGnRH-A treatment.

C. Effect of multiple treatment of goldfish with sGnRH-A on the binding parameters of [^{3}H]-spiperone to goldfish pituitary homogenate (March, 1987).

sGnRH-A-treatment significantly increased the numbers of specific [^{3}H]-spiperone binding sites (dopamine/neuroleptic receptors) in the pars distalis over that of vehicle-treated fish, and caused a large but non-significant increase in the number of binding sites in the neurointermediate lobe (Table V-1). The equilibrium dissociation constant (K_a) was unaffected by sGnRH-A treatment.

D. Effects of treatment with sGnRH-A and domperidone on GnRH receptors in the goldfish pituitary.

The displacement curve obtained using pituitaries from control-treated fish indicated the presence of two classes of binding sites for sGnRH-A, a high affinity/low capacity site and a low affinity/high capacity site, as described previously by Habibi et al. (1987a). Displacement curves were also obtained using pituitaries obtained from fish which received either two injections of sGnRH-A, 12 h apart, or a single injection of domperidone. Treatments with sGnRH-A or domperidone were without affect on the binding affinity of high or low affinity sites, but significantly affected GnRH receptor capacity in the goldfish pituitary (Table V-2). Both sGnRH-A and domperidone significantly increased the capacity of the high affinity binding site, but reduced the capacity of the low affinity binding site compared to the controls (Table V-2).

DISCUSSION

Our findings indicate that the capacity of the goldfish pituitary to release GtH in response to domperidone increases with advancing seasonal gonadal recrudescence (from January to March); however, there was no significant change in the sensitivity of the serum GtH response to domperidone. Casual examination of the raw data did not reveal any differences between male and female fish in their serum GtH responses to domperidone. Following gonadal recrudescence the maximum serum GtH response to domperidone declined and remained stable in post-ovulatory fish. The present findings are consistent with those reported by Sokolowska et al., (1985) demonstrating that the magnitude of the serum GtH response to GnRH, pimozide, or their combination was directly related to the stage of gonadal development of the fish. A previous study showed that when administered alone domperidone was more effective than pimozide in elevating serum concentrations of GtH in goldfish (this thesis, Chapter II).

Our data indicate that multiple treatment of goldfish with sGnRH-A significantly increased the magnitude of the serum GtH response to domperidone compared with that of vehicle-treated fish. GnRH, by stimulating GtH release, may promote gonadal development. Furthermore, there is evidence that GnRH potentiates the action of dopamine antagonists like domperidone (this thesis, Chapter II). The present data demonstrate a correlation between seasonal changes in circulating levels of GtH and seasonal changes in the magnitude of the serum GtH response to domperidone, which is also potentiated by sGnRH-A.

In goldfish, there is evidence that increased serum concentrations of GtH may be associated with changes in the intensity of neuronal GnRH

input to the pituitary; Yu, Nahorniak, Peter et al. (1987) have shown that in female goldfish maintained at 10 °C, hypothalamic and pituitary GnRH content decreased during ovarian recrudescence. Furthermore, Yu et al. (1987) have shown a short-term decrease in the GnRH content of the hypothalamus and pituitary (as well as olfactory bulbs and telencephalon) in fish undergoing spontaneous ovulation; ovulation is preceded by a pre-ovulatory surge in serum concentrations of GtH. These findings suggest that in goldfish increased neuronal GnRH stimulation of the pituitary occurs concomitantly with decreased hypothalamic and pituitary GnRH content, resulting in increased circulating levels of GtH. Seasonal changes in GnRH stimulation of gonadotrophs may induce changes in the GtH secretory potential of the pituitary which may explain, in part, seasonal alterations in the serum GtH response to domperidone. Preliminary evidence indicates that the increase in serum concentrations of GtH in response to i.p. injection of domperidone into goldfish is accompanied by decreased hypothalamic and pituitary GnRH content (K.L. Yu, personal communication). The present findings suggest that domperidone increases serum concentrations of GtH by blocking pituitary dopamine receptors and perhaps also in part by increasing GnRH neuronal stimulation of the pituitary.

The present findings indicate that the number of pituitary GnRH receptors is affected by treatment with either sGnRH-A or domperidone; while the treatments increased the capacity of the high affinity sites, they reduced the capacity of the low affinity binding sites. There is a correlation between seasonal changes in the numbers of high- and low-affinity GnRH receptors and seasonal changes in the serum GtH response to i.p. injections of GnRH-analogues (Habibi, Van der Loo,

Marchant & Peter, 1987b); the function of the low affinity sites is not clear. In the present study, the observed increase in the capacity of the high affinity GnRH receptors following treatments with sGnRH-A and domperidone is in accord with the increased sensitivity of the serum GtH response in goldfish following treatments with sGnRH-A and domperidone (Experiments A, B). Nonetheless, it is interesting that i.p. injection of domperidone caused a greater increase in the numbers of high-affinity GnRH receptors than did i.p. injections of sGnRH-A; yet, serum concentrations of GtH in domperidone treated fish were lower than in sGnRH-A treated fish (Table V-2). This phenomenon requires further investigation.

The results of this study confirm and extend our previous demonstration that sGnRH-A potentiates the serum GtH response to domperidone (this thesis, Chapter II). The present study demonstrates that repeated i.p. injection of sGnRH-A into goldfish increases the number of pituitary dopamine/neuroleptic receptors, suggesting that the magnitude of the serum GtH response to domperidone may be related, in part, to the number of these receptors. At present, the mechanism by which sGnRH-A affects dopamine/neuroleptic receptor numbers in the goldfish is not clear. However, evidence based on studies on mammals indicates that the numbers of brain and pituitary dopamine receptors are inversely related to the neural supply of dopamine (for review see Seeman, 1981). More specifically, a decrease in the concentration of dopamine in hypothalamic portal blood is linked to increased numbers of pituitary dopamine receptors (Heiman & Ben-Jonathan, 1982; 1983). Seasonal changes in dopamine input to the goldfish pituitary could induce alterations in dopamine receptor numbers which could, in part,

account for seasonal changes in the serum GtH response to domperidone; there is, however, little direct evidence regarding seasonal changes in dopamine input to the teleost pituitary. In mammals, modulation of dopamine neuronal function and direct blockade of dopamine receptors, by dopamine antagonists can induce increased numbers of dopamine receptors leading to dopamine hypersensitivity (Seeman, 1981); experiments on changes in the numbers of pituitary dopamine/neuroleptic receptors in goldfish treated with domperidone have not been undertaken in this study as large amounts of domperidone are retained in the pituitary as long as 96 h after a single injection (this thesis, Chapter II). In this context, domperidone bound to pituitary receptors would tend to interfere with accurate determinations of receptor binding parameters. The results of the present study confirm and extend our previous findings that the density of dopamine/neuroleptic receptors is greater in the neurointermediate lobe than in the pars distalis of the goldfish (this thesis, Chapter IV)

In summary, we present evidence for seasonal variation in the magnitude of the serum GtH response to domperidone, a dopamine antagonist, and provide information on the interaction of GnRH and dopamine in the regulation of GtH release in goldfish. Our findings suggest that this interaction partly involves changes in the numbers of pituitary receptors for GnRH and dopamine.

Table V-1. Influence of [DArg^6, Trp^7, Leu^8, Pro^9-N-ethylamide]-LHRH (sGnRH-A) treatment on the specific binding of [^{3}H]-spiperone to goldfish pituitary homogenate.

Tissue	Ka (10^5 M^{-1})		Capacity (nmol/mg protein)	
	Veh	sGnRH-A	Veh	sGnRH-A
PD	1.92 ± 0.28	1.63 ± 0.09	15.34 ± 2.06	25.22 ± 1.24
	(1.27 - 2.56)	(1.42 - 1.83)	(10.58 - 20.09)	(22.35 - 28.08)
NIL	3.34 ± 0.71	3.67 ± 0.90	46.14 ± 8.26	69.11 ±12.94
	(1.69 - 4.98)	(1.59 - 5.74)	(27.05 - 65.22)	(39.21 - 99.00)

Values are means ± S.E.M. as calculated by LIGAND from 1 experiment. Values in parentheses indicate 95 % confidence intervals based on the mean.

Table V-2. Effects of treatment with sGnRH-A and domperidone on the pituitary GnRH receptor affinity (equilibrium association constant, K) and capacity (R) in the goldfish.

	High Affinity Site		Low Affinity Site	
	($\times 10^{10}$ $K1_M^{-1}$)	(fmol / mg protein) R1	($\times 10^{7}$ $K2_M^{-1}$)	(pmol / mg protein) R2
Vehicle	2.52 ± 0.6 (1.08-3.96)	35.62 ± 2.6 (29.38-41.86)	2.26 ± 0.3 (1.54-2.98)	27.5 ± 0.48 (26.3-28.65)
sGnRH-A	1.52 ± 0.36 (0.656-2.38)	68.9 ±7.6 (50.66-87.14)	2.82 ± 0.14 (2.48-3.15)	21.97 ± 0.56 (20.62-23.31)
DOM	1.66 ± 0.68 (0.03-3.29)	78.81 ± 17.2 (37.52-120.09)	2.59 ± 0.42 (1.58-3.59)	8.22 ± 1.28 (5.14-11.29)

Values were estimated using a computerized Scatchard analysis (LIGAND) of displacement curves. Results to sGnRH-A and domperidone (DOM) injection were obtained using 2 and 1 displacement curves, respectively. All incubations were carried out in triplicate to sGnRH-A concentrations, 10^{-12}-10^{-6} M, using pituitary homogenate from 40 fish for each displacement experiment. 95 % C.I., 95 % confidence intervals.

Figure V-1: Seasonal variation in serum concentrations (ng/ml) of gonadotropin (GtH) in goldfish 24 h after i.p. injection of domperidone plotted as a function of domperidone dose.

Values are means $\pm$ S.E.M. (n=7 to 10). The minimum dose of domperidone required to significantly increase serum GtH levels was 1, 1, 5, 10, 5, and 1 umol/kg body weight in January, March, May, July, September and December, respectively; data were subjected to analysis of variance and Duncan's multiple range test.

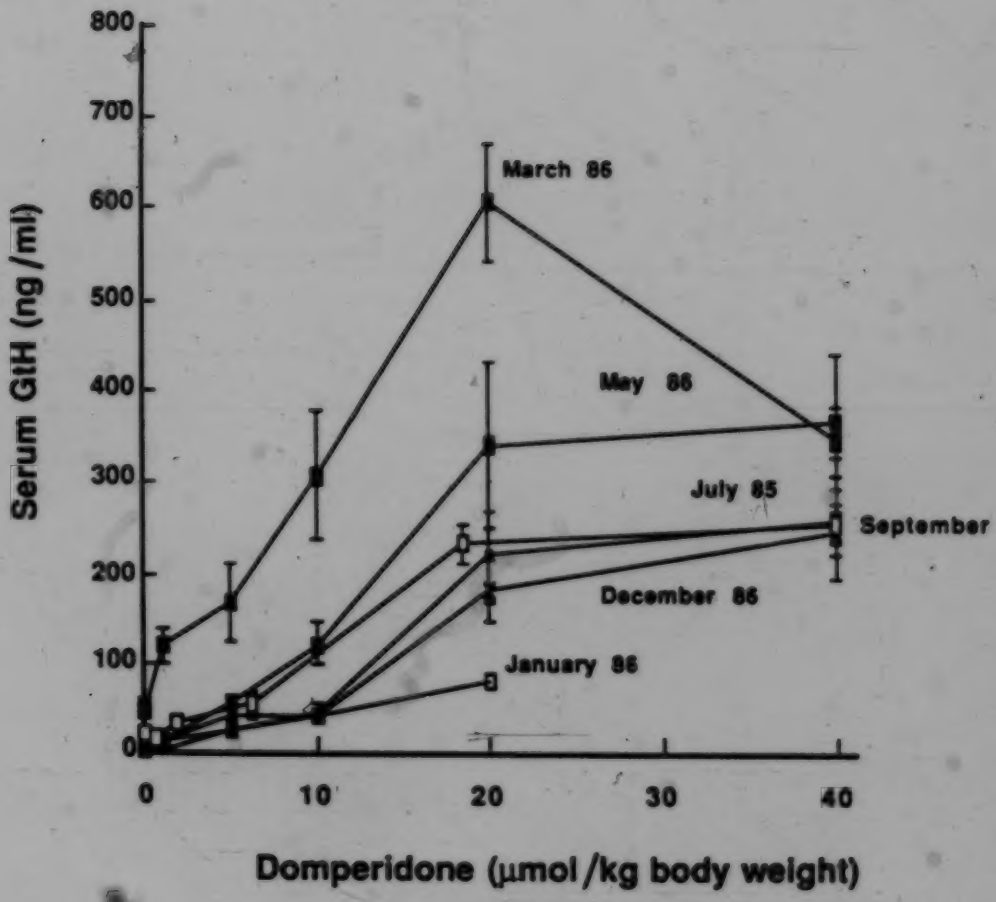

800
700
600
500
400
300
200
100
0
Serum GtH (ng/ml)
March 86
May 86
July 85
September
December 86
January 86
0
10
20
30
40
Domperidone (µmol/kg body weight)

Figure V-2: Seasonal changes in simulated ambient temperature, photoperiod, and serum concentrations of gonadotropin (GtH) in goldfish.

Seasonal changes in serum concentrations of GtH (ng/ml; values are means ± S.E.M.; n=7 to 10) in vehicle-injected fish, and simulated natural ambient photophase and temperature; horizontal bars indicate values not significantly different from each other (analysis of variance and Duncan's multiple range test).

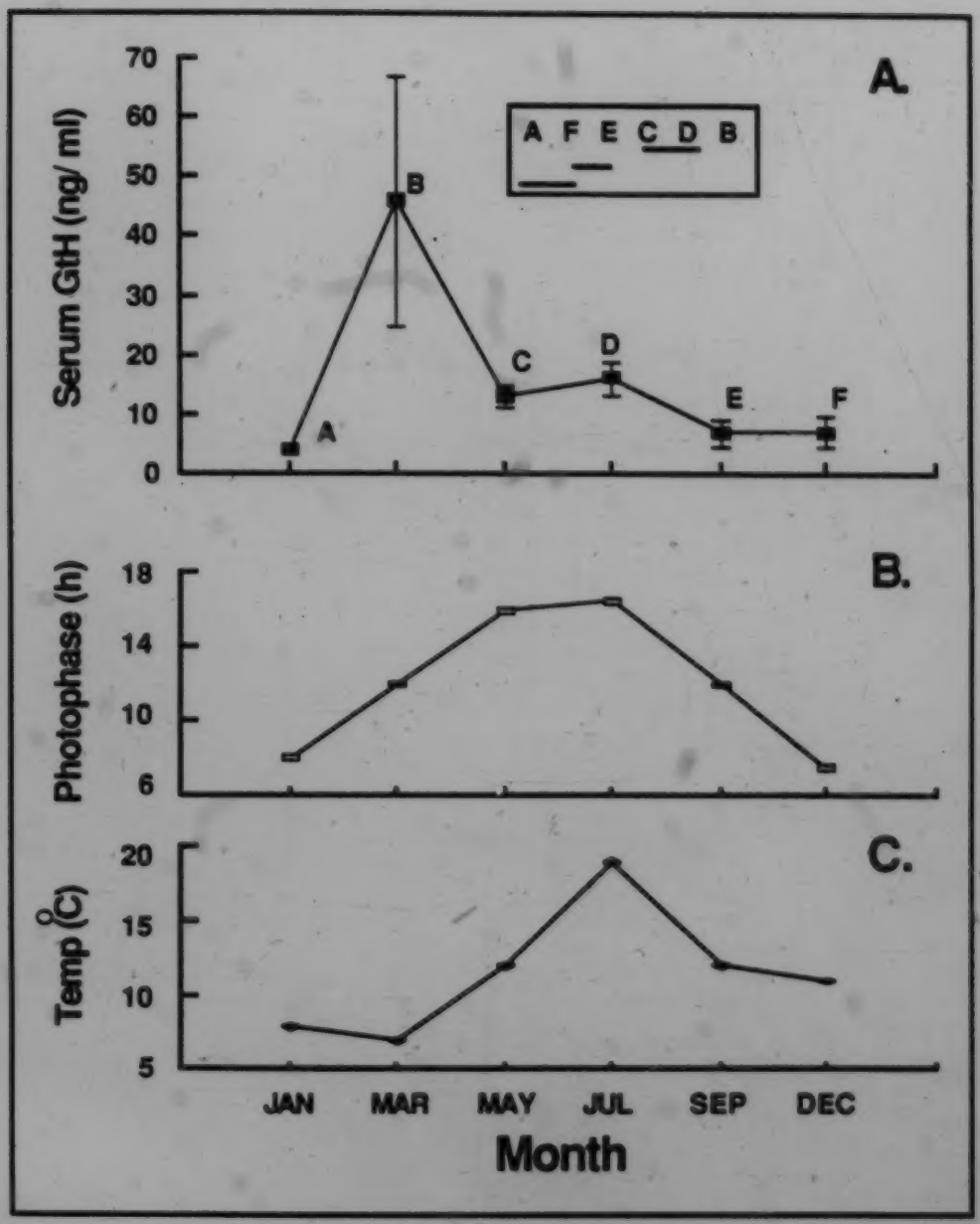

A.
Serum GtH (ng/ ml)
70
60
50
40
30
20
10
0
A F E C D B
A
B
C
D
E
F
B.
Photophase (h)
18
14
10
6
C.
Temp (°C)
20
15
10
5
JAN
MAR
MAY
JUL
SEP
DEC
Month

Figure V-3: Effect of multiple i.p. injections of [D-Arg^{6}, Trp^{7}, Leu^{8}, Pro^{9}-NEt]-LHRH (sGnRH-A) on serum concentrations of gonadotropin (GtH) in goldfish.

Serum concentrations of gonadotropin (GtH; ng/ml) in goldfish 24 h prior to or following i.p. injections of vehicle or [D-Arg^{6}, Trp^{7}, Leu^{8}, Pro^{9}-NEt]-LHRH (sGnRH-A), plotted as a function of treatment; injections were 48 h apart. Values are means $\pm$ S.E.M. (n=7-10); data were subjected to analysis of variance and Duncan's multiple range test. *indicates values significantly ($p<0.05$) different compared with pre-injection values.

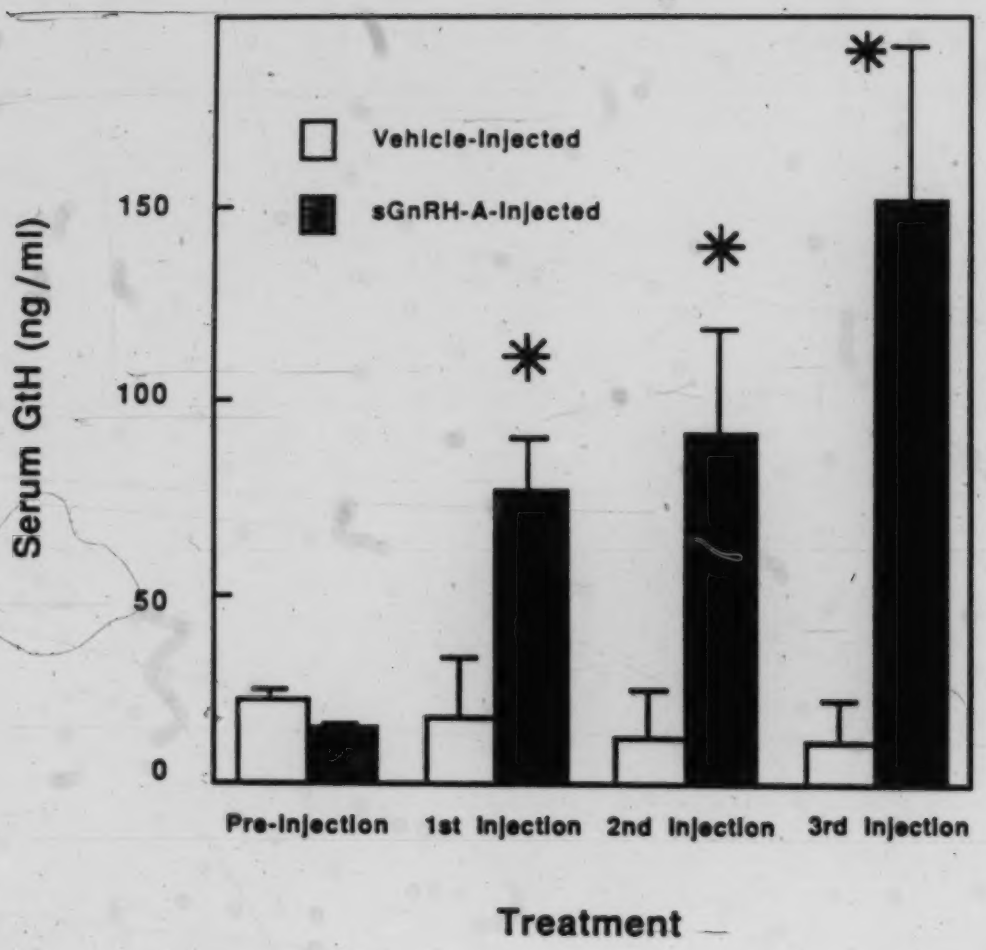
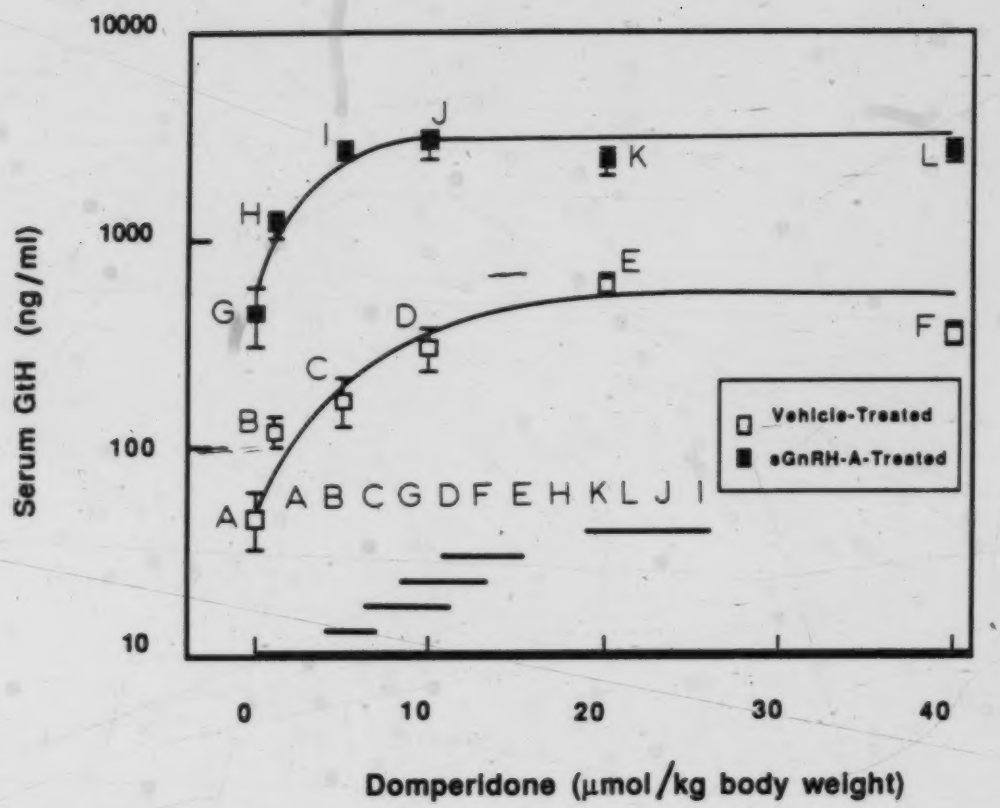

Vehicle-injected
sGnRH-A-injected
Serum GtH (ng/ml)
150
100
50
0
Pre-injection
1st injection
2nd injection
3rd injection
Treatment

Figure V-4: Effect of multiple i.p. injections of [D-Arg^{6}, Trp^{7}, Leu^{8}, Pro^{9}-NEt]-LHRH (sGnRH-A) on the serum gonadotropin (GtH) response to domperidone.

Serum concentrations of GtH (ng/ml) in goldfish 24 h after an i.p. injection of vehicle or a given dose of domperidone; fish were pre-treated with 3 i.p. injections, 48 h apart, of either vehicle or sGnRH-A. Values are means $\pm$ S.E.M. (n=7 to 10) plotted as a function of domperidone dose (umol/kg body weight). Data were analyzed by analysis of variance and Duncan's multiple range test. Horizontal bars indicate values not significantly ($p<0.05$) different from each other.


10000
1000
100
10
Serum GtH (ng/ml)
A
B
C
D
E
F
G
H
I
J
K
L
A B C G D F E H K L J I
Vehicle-Treated
sGnRH-A-Treated
0
10
20
30
40
Domperidone (μmol/kg body weight)

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VI. GENERAL DISCUSSION

Studies of the goldfish pituitary dopamine receptor were described in chapters II through V; this chapter presents a summary and general discussion of the findings.

In vivo (Chapter II) and in vitro (Chapter III) studies of dopamine and dopamine antagonist manipulation of GtH release suggest that the goldfish pituitary dopamine receptor is a relatively low affinity receptor, functional at ligand concentrations of between 10 nM and 10 uM. This is further supported by evidence for the existence of a low affinity dopamine/neuroleptic binding site in the goldfish pituitary, as assessed by the in vitro radioreceptor assay (Chapter IV). In comparison with our data on the dopamine receptor, GnRH ligands bind to the goldfish pituitary GnRH receptor at nM to sub-nM concentrations (Habibi, Peter, Sokolowska et al., 1987) and are effective in stimulating GtH release in vitro at similar concentrations (Peter, Habibi, Marchant & Nahorniak, 1987), and in vivo at nmole/kg body weight doses (Chapter II).

Dopamine receptor-mediated inhibition of GtH release in goldfish is specific, as indicated by the in vivo action of dopamine antagonists from different chemical classes (Chapter II); as well, there is a small but significant stereospecific reversal, by isomers of sulpiride, of endogenous dopamine inhibition of GtH release in vivo. In vitro, stereospecificity of sulpiride antagonism of dopamine inhibition of GtH release or stereospecificity of sulpiride interaction with the dopamine/neuroleptic binding site are difficult to demonstrate. In mammals, sulpiride and other neuroleptics commonly cross the blood:brain barrier to block central as well as peripheral dopamine receptors; it is

possible that sulpiride also crosses the goldfish blood:brain barrier to block central dopamine receptors and thereby indirectly influence GtH release from the pituitary. *In vitro* data suggest only minor stereospecificity of apomorphine inhibition of GtH or α-MSH release (Chapter II).

Domperidone, compared with other dopamine antagonists tested, has unique properties which make it most suitable for the study of the goldfish pituitary dopamine receptor. Domperidone injected i.p. is excluded from the goldfish brain (Chapter II) and is one of the most potent of the dopamine antagonists tested for elevation of serum concentrations of GtH. The goldfish pituitary preferentially accumulates i.p. injected [^{3}H]-domperidone; as well, the time course of variation in pituitary levels of [^{3}H]-domperidone, following i.p. injection of [^{3}H]-domperidone, is similar to the time course of the serum GtH response to i.p. injected domperidone (Chapter II). *In vitro* data indicate that dopamine and domperidone act directly on the pituitary to rapidly alter GtH and α-MSH release. Together, these *in vivo* and *in vitro* data support the concept that domperidone injected i.p. into goldfish acts rapidly, following binding to pituitary dopamine/neuroleptic receptors, to increase GtH (and α-MSH) release.

Domperidone actions in mammals are mediated exclusively through dopamine (D2) receptors. Domperidone is structurally related to pimozide, a selective dopamine receptor antagonist which has a similar potency in elevating serum concentrations of GtH in the goldfish *in vivo*. Pimozide was reported to modulate the stimulatory action of GnRH and a synthetic calcium ionophore on GtH release, and, it was suggested that pimozide's actions were the result of calmodulin inhibition (Conn,

Rogers & Sheffield, 1981). This proposed mechanism for pimozide appears inappropriate for the stimulatory action of pimozide on GtH secretion in goldfish. Also, pimozide has been widely used and evaluated as a selective dopamine receptor antagonist and effective neuroleptic (Seeman, 1981; for review see Pinder, Brogden, Sawyer et al., 1976). Although the possibility of actions on calmodulin cannot be excluded, the best explanation for the actions of both domperidone and pimozide in the goldfish is that the effects are mediated through a dopamine/neuroleptic receptor.

Dopamine inhibition of α-MSH release in teleosts has been previously suggested (Olivereau, 1978; Olivereau, Olivereau & Lambert, 1987). Our findings support this hypothesis, as dopamine and apomorphine reversibly inhibit α-MSH release from goldfish NIL fragments; as well, domperidone antagonizes this inhibitory action of dopamine (Chapter III). Dopamine inhibition of α-MSH release is well documented in amphibians (for review see Tonon, 1984), and in mammals it has been confirmed that dopamine inhibits α-MSH release by acting through a dopamine D2 receptor subtype on melanotrophs (Cote, Eskay, Frey et al., 1982). Involvement of dopamine in regulating α-MSH release from the goldfish NIL is further supported by the existence of large numbers of dopamine/neuroleptic receptors in the NIL; the population of these receptors in the NIL is greater than that observed in the PD (Chapter V). In general, dopamine inhibition of GtH and α-MSH release in vitro are similar in that both spontaneous and releasing-factor stimulated hormone release are affected; as well, dopamine agonists and antagonists have roughly similar potencies in altering GtH and α-MSH release in vitro. Also, dopamine agonists and antagonists alter GtH and α-MSH

release *in vitro* in a concentration range and rank order similar to their inhibition of [^{3}H]-spiperone binding to the goldfish pituitary *in vitro*.

A preliminary investigation of the interaction of GnRH and dopamine action on regulation of GtH release was also carried out. GnRH is a potent stimulator of GtH release *in vivo* (Chapter II) and *in vitro* (Chapter III). Earlier studies indicated that domperidone and other dopamine antagonists potentiate the actions of GnRH *in vivo* in goldfish (for review see Peter, Chang, Nahorniak *et al.* (1986)). The present findings regarding the combined actions of domperidone and GnRH support and extend this concept. Underlying this is the demonstration that antagonism of endogenous dopamine inhibition by dopamine receptor antagonists permits greater expression of GnRH action. On the other hand GnRH, by some mechanism, permits greater expression of the action of domperidone. In addition, evidence is presented which shows that multiple i.p. injections of GnRH into goldfish not only increases the maximum serum GtH response to domperidone by 4 to 5 fold, but also causes a large, significant increase in PD dopamine/neuroleptic receptor numbers (Chapter V); receptor numbers were increased, non-significantly, in the NIL. As well, GnRH-receptor numbers were increased in the PD by multiple i.p.injections of GnRH as well as by multiple domperidone injections; the possible existence of GnRH-receptors in the NIL was not explored.

There are at least three possible explanations for GnRH-mediated increases in goldfish pituitary dopamine/neuroleptic receptor numbers. Firstly, GnRH acts on the pituitary to release GtH, which stimulates the synthesis and release of gonadal steroids by the gonads and these

steroids in turn have a positive feedback effect on the pituitary. In a preliminary study, multiple i.p. injections of testosterone into sexually recrudescing goldfish had no effect on the sensitivity or magnitude of the serum GtH response to domperidone (Omeljaniuk & Peter, unpublished results). There is, however, evidence which suggests a positive role of gonadal steroids on GtH release and potentiation of GnRH action (R.E. Peter, personal communication). Secondly, GnRH acts directly on the pituitary to increase receptor numbers. This phenomenon has been demonstrated in rats (Clayton & Catt, 1981), and data in Chapter V suggest that this may also occur in the goldfish. Thirdly, GnRH indirectly antagonizes dopamine inhibition of the pituitary by modulating dopamine neuron activity; there is evidence from mammalian studies which indirectly supports the concept that GnRH in some way may modulate dopamine receptor levels. In circumstances, in mammals, where there is a decrease in dopamine input to a brain or pituitary target site, caused for example by disease or physically/chemically induced-brain lesions, there is a dramatic increase in the number of target site dopamine receptors, accompanied by an increase in the responsivenesss of the tissue to administered dopamine dopamine agonists (dopamine hypersensitivity) (for review see Seeman, 1981). Dopamine hypersensitivity is also observed in mammals and humans exposed to chronic neuroleptic treatment. Thus, in the goldfish, GnRH and dopamine act directly on the pituitary to stimulate and inhibit GtH release, respectively; as well, GnRH may act presynaptically on dopamine neurons to modulate dopamine release, as manifested in elevated pituitary dopamine/neuroleptic receptor numbers; endogenous GnRH would then be permitted to act more intensely on GtH synthesis and secretion.

Conversely, dopamine may modulate GnRH release; domperidone antagonism of dompamine action may facilitate GnRH release as manifested by increased GtH release and pituitary GnRH receptor numbers. Indeed, evidence for changes in goldfish brain and pituitary GnRH content *in vivo*, suggestive of increased GnRH release, has been presented by Yu, Nahorniak, Peter *et al.* (1987). Also, i.p. injection of domperidone reduces pituitary GnRH content, presumably reflecting increased GnRH release (Yu & Peter, unpublished data). In mammals there is evidence which suggests indirect (Jarjour, Handelsman, Raum & Swerdloff, 1986) and direct (Sarkar & Fink, 1981) actions of dopamine on GnRH release.

Presently there is no direct evidence regarding the post-receptor mechanism by which GnRH stimulates GtH release in teleosts. In contrast, there is a large amount of data regarding pituitary GnRH receptors and the post-receptor mechanism(s) through which GnRH stimulates LH release in mammals (for review see Catt, Loumaye, Wynn *et al.*, 1985; also see Chang, McCoy, Morgan & Catt, 1987b). In general, GnRH binds to its pituitary receptor and stimulates LH release through a mechanism which is calcium-dependent, as well as involving rapid phospholipid hydrolysis and activation of protein kinase C (Catt *et al.*, 1985); activation of calmodulin has also been proposed to mediate GnRH action (for review see Conn, McArdle, Andrews & Huckle, 1987). The initial increase in phospholipid metabolites linked to LH release *in vitro* occurs within 15 seconds following stimulation with GnRH (Morgan, Chang & Catt, 1987); increased LH release occurs within 30 seconds of GnRH stimulation (Chang, Graeter & Catt, 1987a). Recent evidence suggests the involvement of voltage-dependent calcium channels in the GnRH post-receptor mechanism; it has been demonstrated that within 10 seconds of GnRH

stimulation of rat gonadotrophs there is a significant increase in cytosolic Ca^{++} (Chang, McCoy, Graeter et al., 1986; Limor, Ayalon, Capponi et al., 1987).

This study provides the first evidence for direct action of TRH on α-MSH release from the teleost pituitary (Chapter III). Our findings support those of a previous study which described the existence of high-affinity and low-affinity TRH binding sites in the goldfish PD and NIL (Burt & Ajah, 1984). In mammals TRH stimulates the release of TSH and prolactin from the PD. In comparison, TRH is devoid of TSH-releasing activity in lower vertebrates but does stimulate prolactin and α-MSH release (for review see Jackson, 1980). Evidence regarding TRH mechanism(s) of action originates largely from mammalian studies, and these mechanisms have many similarities with several proposed GnRH post-receptor mechanisms including dependence on calcium and nucleotide-binding proteins, stimulation of cytosolic calcium mobilization (Ramsdell & Tashjian, 1985) and perhaps also rapid phospholipid hydrolysis (Aub, Frey, Sekura & Cote, 1986).

The mechanism of action by which dopamine inhibits GtH and α-MSH release in the goldfish is presently unknown; but, these effects may be explained by the action of dopamine on calcium and phospholipid-dependent pathways as suggested for dopamine inhibition of spontaneous and releasing-factor stimulated prolactin (Cronin, 1982) and α-MSH (Cote, Eskay, Frey et al., 1982) release in mammals. Examples of calcium channel involvement in dopamine action include inhibition of prolactin secretion from rat lactotrophs (Judd, Koike, Schettini et al., 1985; Login, Judd & MacLeod, 1986) and α-MSH from mouse melanotrophs (Taraskevich, Tomiko & Douglas, 1986). The involvement of

melanotrophs (Taraskevich, Tomiko & Douglas, 1986). The involvement of calcium channels with hormone release has also been suggested, on the basis of changes in pituitary cell membrane voltage (Cota, 1986), which may be attributed to specific dopamine D2 ligand stimulation (Israel, Jaquet & Vincent, 1985). There is no information on the post-receptor mechanism by which dopamine inhibits GtH release in goldfish. However, an early study using lactotrophs from a teleost (alewife; Alosa pseudoharengus) demonstrated that dopamine at concentrations of 10 nM and 1 uM slowed or abolished, respectively, spontaneous membrane voltage fluctuations in a reversible fashion (Taraskevich & Douglas, 1978). Dopamine and its agonists have also been shown to specifically inhibit TRH action; systemic pretreatment with piribedil, a dopamine agonist, significantly reduced TRH-stimulated prolactin release in rats (Hylka, Forman, Sonntag & Meites, 1986). As well, dopamine (500 nM) inhibited TRH stimulation of prolactin release from bullfrog (Rana catesbiana) hemi-pituitaries in vitro (Seki & Kikuyama, 1986); dopamine ligands also modify TRH stimulation of α-MSH release from frog (R. ridibunda) intermediate lobe fragments in vitro (Tonon, 1984).

Currently, phosphotidylinositide metabolism is suggested as an important component in the dopamine post-receptor mechanism. In an in vitro study using rat pituitary glands, dopamine inhibited the incorporation of ^{32}Pi into phosphotidylinositides; this inhibitory action of dopamine was reversible and was antagonized by the dopamine receptor antagonists haloperidol and pimozide (Canonico, Valdenegro, MacLeod et al., 1983). In vivo experiments demonstrated that i.p. injection of bromocryptine inhibited phosphoinositide turnover (Canonico et al., 1983).

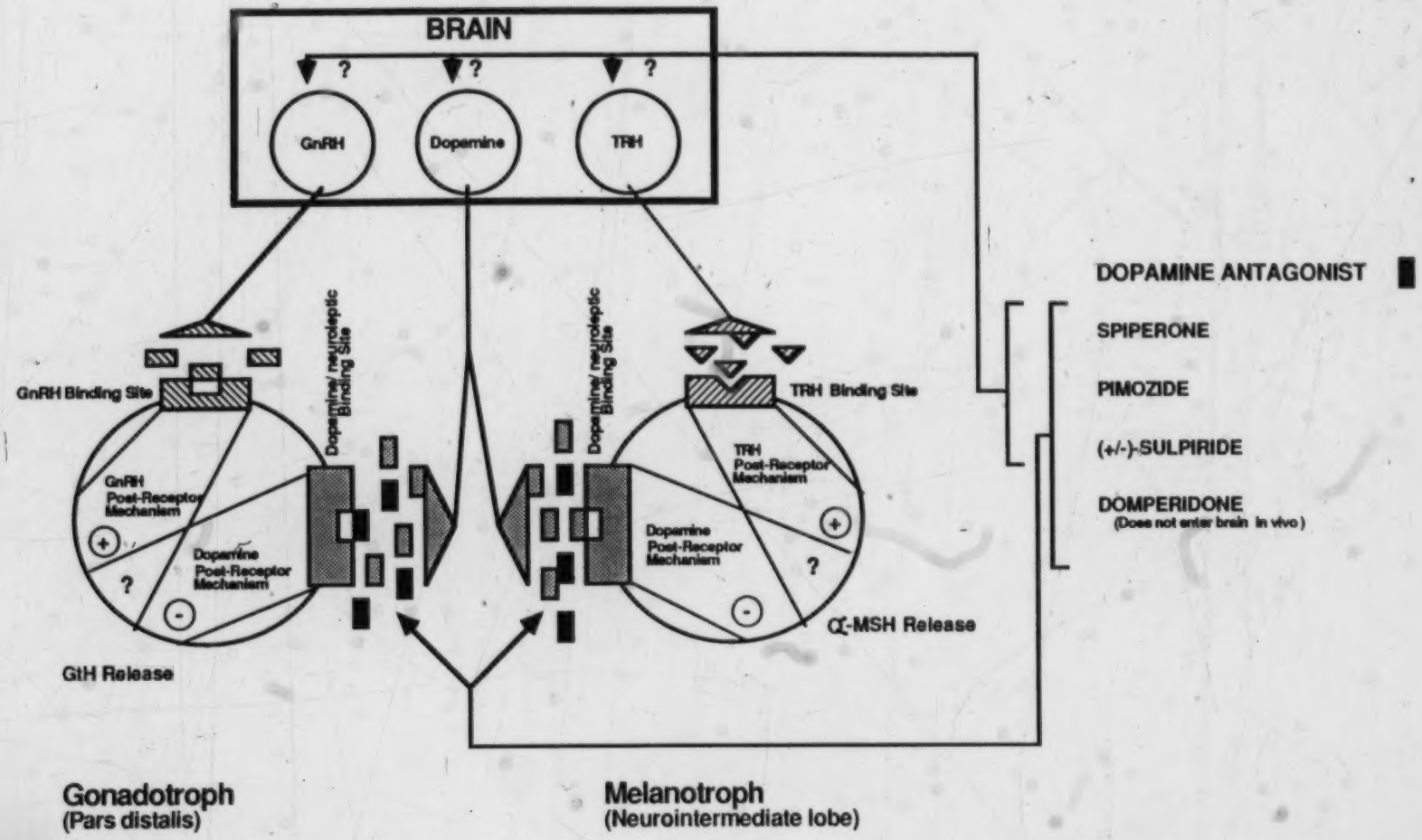
Other post-receptor mechanisms may be involved in dopamine inhibition of pituitary hormone release. Involvement of adenyl cyclase was suggested based on studies of short-term cultures of melanotrophs from the intermediate pituitary of the rat; the production of a proopiomelanocortin-like material and secretion of an α-MSH-like material were inhibited by bromocryptine, a dopamine agonist, and quinpirole, a specific dopamine D2-receptor agonist, while SCH 23390, a selective D1-antagonist, had no effect; spiperone, 8-bromo-cAMP and YM-09151-2, a selective D2 antagonist, antagonized the actions of bromocryptine (Beaulieu, Felder & Kebabian, 1982). In superfused rat anterior pituitary cells, prolactin release was stimulated and inhibited by TRH and dopamine, respectively; dopamine also inhibited the stimulatory action of forskolin and 8-bromo-cAMP (Delbeke & Dannies, 1985). However, in a similar study using purified lactotrophs from pituitary tumours it was found that the decrease in cAMP levels accompanying dopamine treatment was not necessary for dopamine inhibition of prolactin release (Delbeke, Scammel, Martinez-Campos *et al.*, 1986). Thus the involvement of cAMP in the post-receptor mechanism by which dopamine inhibits prolactin release is at best unresolved. Calmodulin has also been inplicated as being part of the dopamine post-receptor mechanism (for citations see Means & Chafoulas, 1982).

In summary, the data in this thesis indicate the existence of a goldfish pituitary dopamine receptor. The binding characteristics of the binding site satisfy criteria for a receptor, and binding affinity and specificity are closely correlated with biological responses; these characteristics resemble some of those of the classical dopamine D2 receptor subtype. This receptor mediates dopamine inhibition of GtH and

α-MSH release from the goldfish pituitary; the post-receptor mechanism for dopamine action in this system is as yet unknown. A schematic summary of our major findings is shown in Figure VI-1.

Figure VI-1. Schematic summary of the proposed interactions of hypothalamic releasing factors and dopamine ligands in regulation of gonadotropin (GtH) and α -melanocyte-stimulating hormone (α -MSH) release from the pituitary of the goldfish, Carassius auratus.

In this proposed model, the hypothalamic releasing factors gonadotropin releasing-hormone (GnRH) and thyrotropin releasing-hormone (TRH) bind to, and stimulate, their receptors to initiate the post-receptor mechanisms leading to the release of GtH and α -MSH, respectively; dopamine binds to its dopamine/neuroleptic receptor initiating its mechanism of action to inhibit hormone release and to modulate the action of hypothalamic releasing factors. Dopamine antagonists inhibit dopamine action by blocking dopamine/neuroleptic receptors; some dopamine antagonists may also have central effects.


BRAIN
?
?
?
GnRH
Dopamine
TRH
GnRH Binding Site
Dopamine/ neuroleptic Binding Site
Dopamine/ neuroleptic Binding Site
TRH Binding Site
GnRH Post-Receptor Mechanism
Dopamine Post-Receptor Mechanism
TRH Post-Receptor Mechanism
Dopamine Post-Receptor Mechanism
+
?
-
+
?
-
GtH Release
α-MSH Release
DOPAMINE ANTAGONIST
SPIPERONE
PIMOZIDE
(+/-)-SULPIRIDE
DOMPERIDONE
(Does not enter brain in vivo)
Gonadotroph
(Pars distalis)
Melanotroph
(Neurointermediate lobe)

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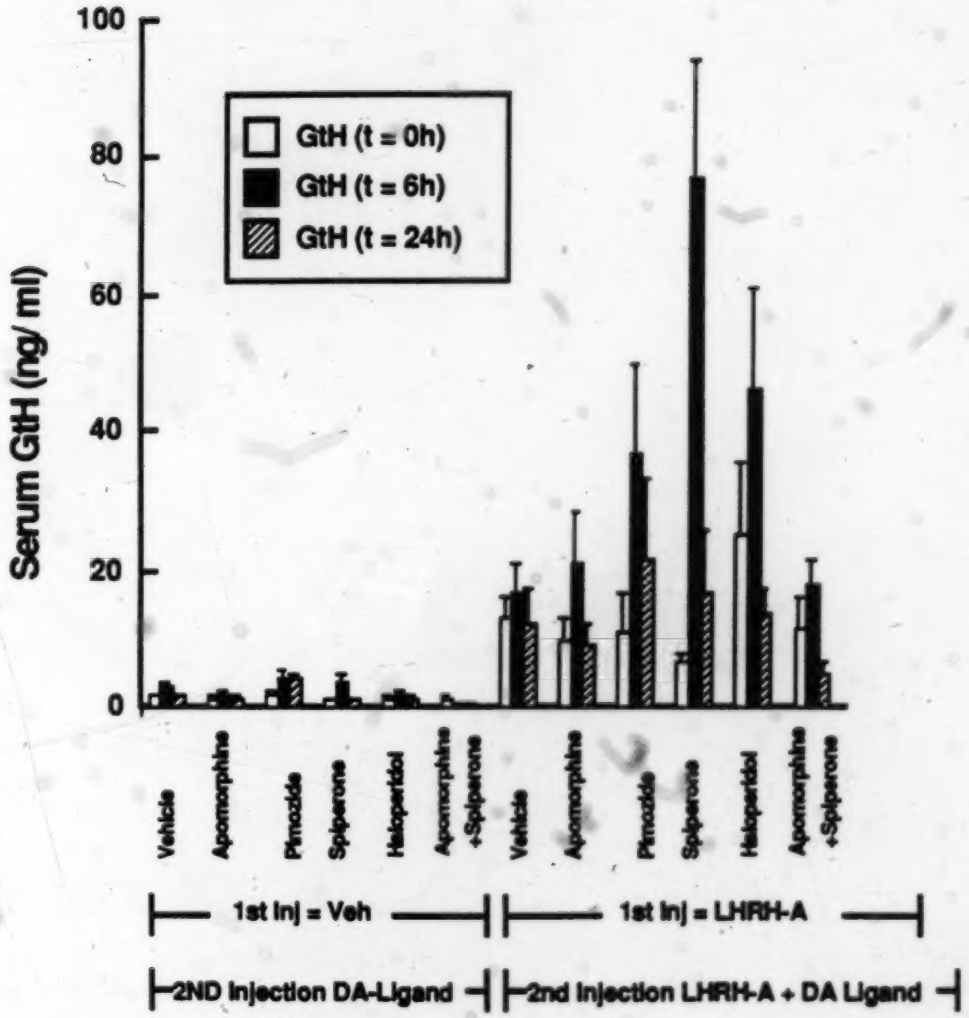
APPENDIX I.

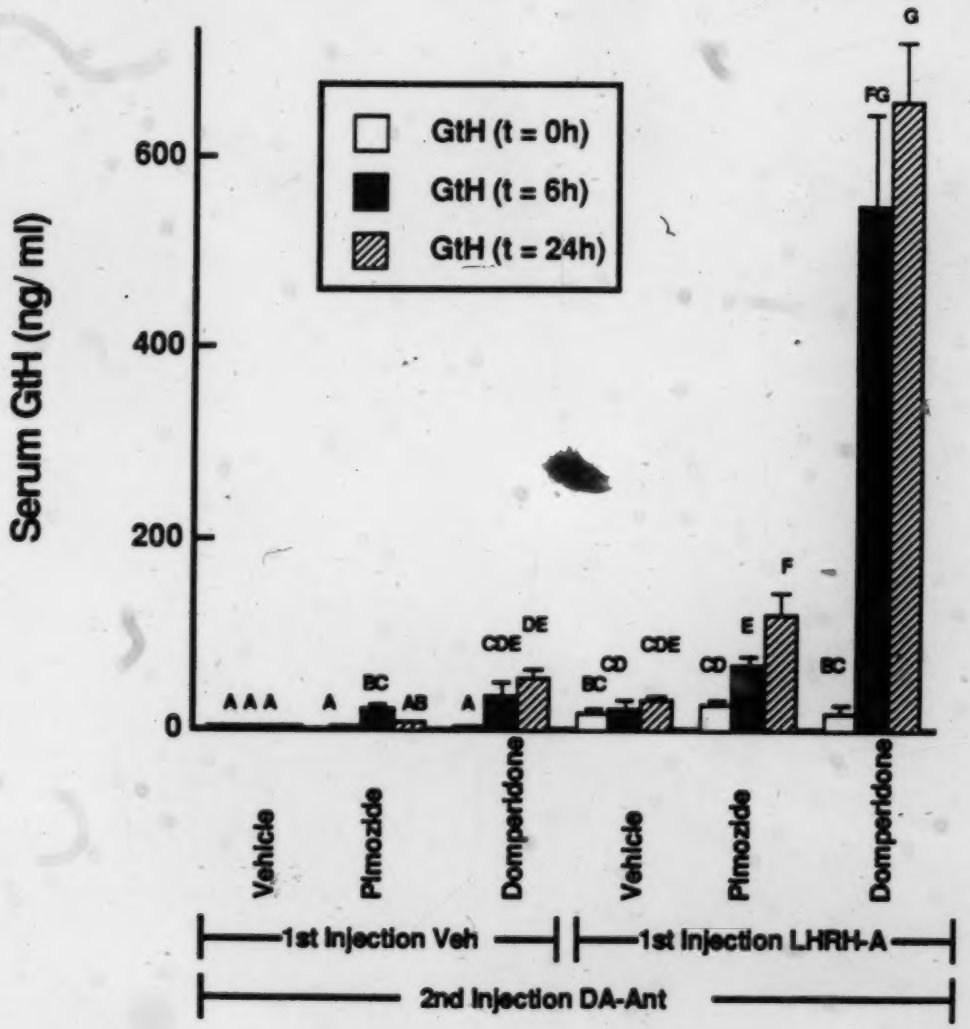
A. Comparison of the effects of various dopamine ligands on the release of GtH and on the action of a GnRH analogue in the goldfish in vivo.

Male and female goldfish (sexually recrudescing; March, 1984) were maintained at 12 ^{o}C and 12 h photoperiod. At t = -6h fish were first injected i.p. with saline or LHRH-A (0.1 ug/g BW); at t=0h fish were blood sampled and injected with saline or a dopamine ligand in suspension (10 ug/g BW) or a combination of LHRH-A and dopamine ligand in a volume of 10 ul/g BW; goldfish were subsequently blood sampled at t = 6h and 24h. Of the dopamine antagonists tested alone, pimozide caused the greatest increases in serum concentrations of GtH at t = 6h and 24h; in contrast, spiperone caused the largest potentiation of LHRH-A action, at 6h (Figure 1). Only pimozide and spiperone potentiated LHRH-A action either at t = 6h or t = 24h. Apomorphine inhibited the action of spiperone alone, or in combination with LHRH-A (Figure 1). Values are means $\pm$ S.E.M. (n=6 to 10). Data were analyzed using a Mann-Whitney U-test at the $p < 0.05$ level of significance; for clarity, statistical significances are not indicated. In another study (September, 1983) using a similar protocol, pimozide and spiperone potentiated LHRH-A action at t = 6h and 24h; apomorphine inhibited the action of pimozide and spiperone (data not shown).


100
80
60
40
20
0
Serum GtH (ng/ ml)
GtH (t = 0h)
GtH (t = 6h)
GtH (t = 24h)
Vehicle
Apomorphine
Pimozide
Spiperone
Haloperidol
Apomorphine +Spiperone
Vehicle
Apomorphine
Pimozide
Spiperone
Haloperidol
Apomorphine +Spiperone
1st Inj = Veh
1st Inj = LHRH-A
2ND Injection DA-Ligand
2nd Injection LHRH-A + DA Ligand

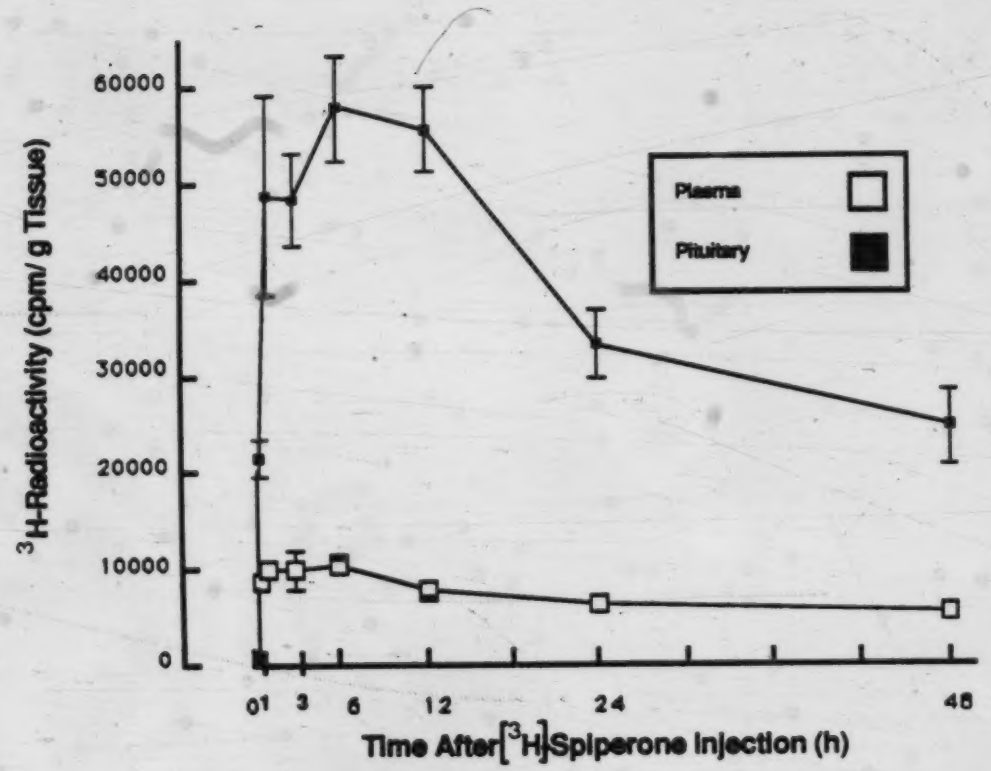
B. Comparison of the effects of pimozide and domperidone on the release of GtH and on the action of a GnRH analogue in the goldfish *in vivo*.

Male and female goldfish (sexually regressed, December 1984) were injected i.p. with vehicle or LHRH-A followed 6 h later with an injection of pimozide (in suspension) or domperidone (in solution) at 10 ug/g BW in a volume of 10 ul/g BW. Alone, pimozide significantly increased serum concentrations of GtH at t = 6h but not at t = 24h; domperidone was effective at t = 6h and t = 24h and was more potent than pimozide at t = 24 h in raising serum concentrations of GtH (Figure 2). Injection of LHRH-A significantly increased serum concentrations of GtH at t = 0, 6, and 24h; pimozide and domperidone both potentiated LHRH-A action at t = 6 and 24h; domperidone was 3 to 5 times as potent as pimozide in potentiating LHRH-A action. Values are means $\pm$ S.E.M. (n = 8 to 10). Data was analyzed by Duncan's multiple range test at the $p < 0.05$ level of significance; values sharing a common letter are not significantly different.


Serum GtH (ng/ml)
600
400
200
0
GtH (t = 0h)
GtH (t = 6h)
GtH (t = 24h)
AAA
A
BC
AB
A
CDE
DE
BC
CD
CDE
CD
E
F
BC
FG
G
Vehicle
Pimozide
Domperidone
Vehicle
Pimozide
Domperidone
1st Injection Veh
1st Injection LHRH-A
2nd Injection DA-Ant

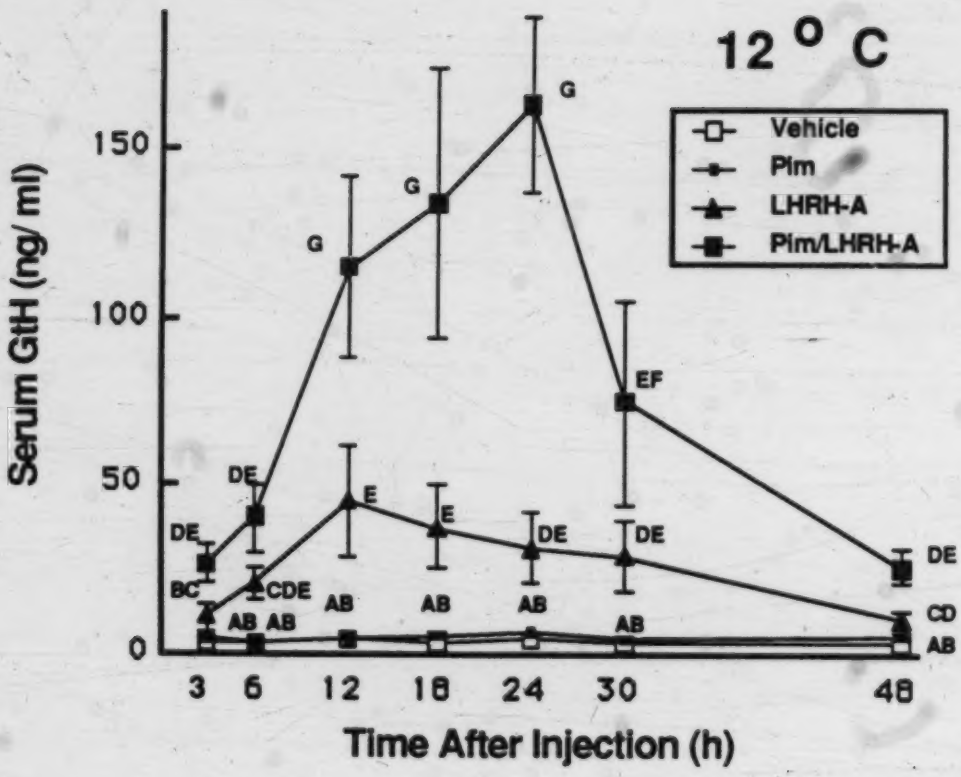
C. Time course of variation in plasma and pituitary [^{3}H]-radioactivity following i.p. injection of [^{3}H]-spiperone into goldfish.

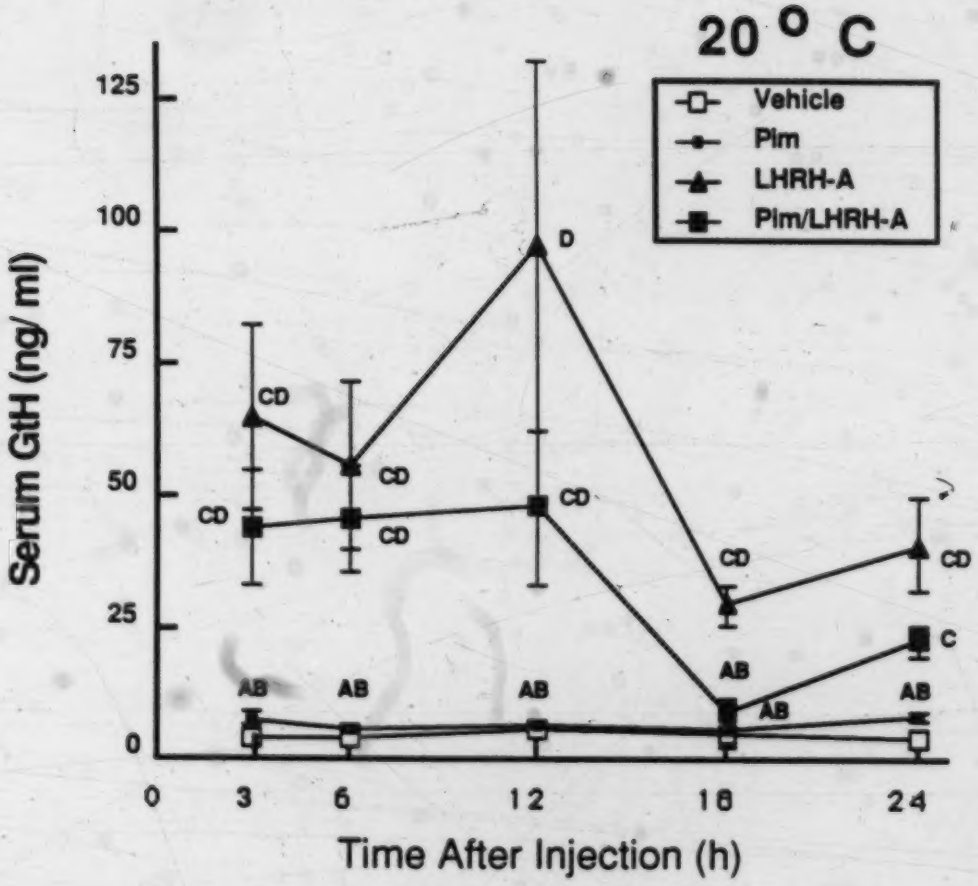
Male and female goldfish (approximately 40 g; sexually regressed; December, 1984) were maintained at 12 ^{o}C and 8 h photoperiod. At t = 0h fish were injected i.p. with [^{3}H]-spiperone (approximately 0.1 uCi; New England Nuclear) in a vehicle of acidified saline (NaCl 0.7 %, sodium metabisulfite 0.1 %, w/v), in a total volume of 200 ul; at various times thereafter fish were blood sampled with heparinized needles/syringes, killed by spinal transection, and the pituitary and plasma counted for ^{3}H-radioactivity as described in Chapter II. Plasma ^{3}H-radioactivity increased to nearly maximal levels at 0.5 h after injection; maximal levels were attained at 6 h after injection; thereafter plasma ^{3}H-radioactivity declined gradually to 48 h (Figure 3). Pituitary ^{3}H-radioactivity increased rapidly following injection of [^{3}H]-spiperone to attain maximal levels 6 h post-injection; thereafter ^{3}H-radioactivity decreased to 48 h. Pituitary ^{3}H-radioactivity, per unit weight of tissue, was 3 to 6 fold greater than plasma ^{3}H-radioactivity at all sampling times. Values are means $\pm$ S.E.M. (n = 8 to 10).


60000
50000
40000
30000
20000
10000
0
^{3}H-Radioactivity (cpm/ g Tissue)
Plasma
Pituitary
0
1
3
6
12
24
48
Time After [^{3}H]Spiperone Injection (h)

D. Time course of action of pimozide and a GnRH-analogue in elevating serum concentrations of GtH in goldfish at 12 °C and 20 °C.

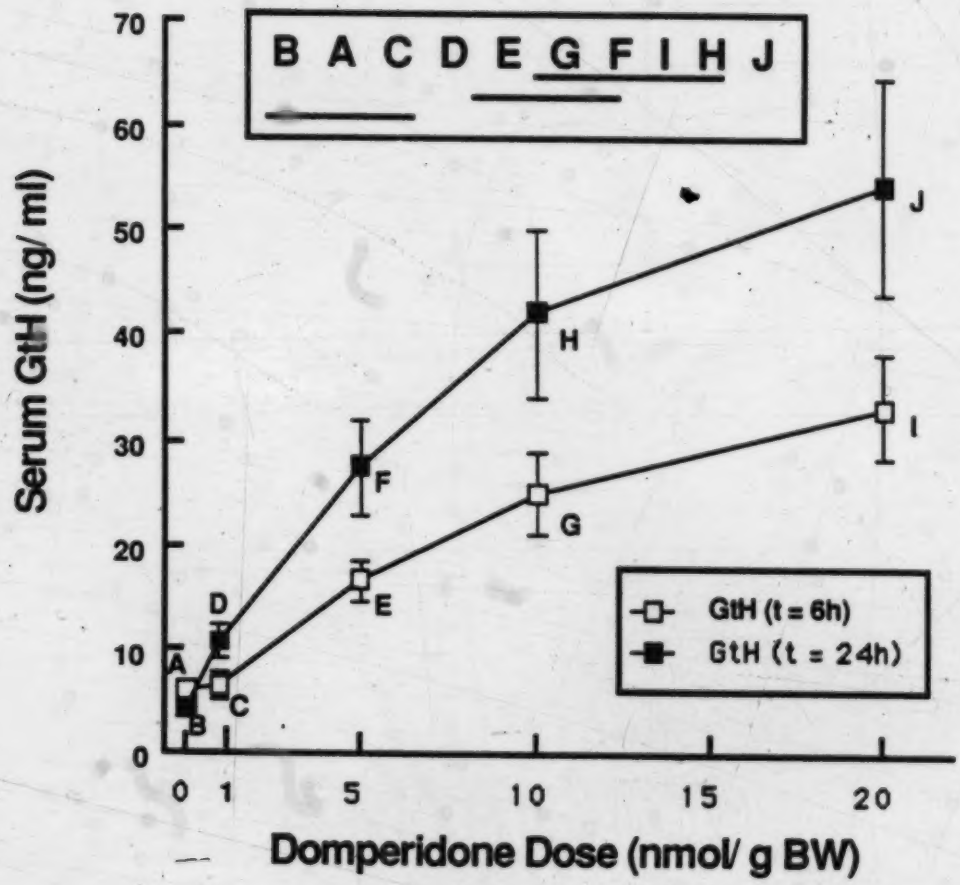
Male and female goldfish (sexually regressed; December, 1984) were maintained at 12 or 20 °C and 8 h photoperiod. Fish were arranged into groups and injected either with pimozide (10 ug/g BW in suspension), or [D-Ala^6]-LHRH (LHRH-A) (0.1 ug/g BW in solution), or a combination of the two in a total volume of 10 ul/g BW in a vehicle of 0.7 % saline; fish were blood sampled at various times after injection and serum was analyzed for GtH content as described in Chapter II. Injection of vehicle or pimozide alone into goldfish had no significant effect on serum concentrations of GtH at either temperature. At 12 °C serum concentrations of GtH were significantly elevated by LHRH-A between 6 and 48 h after injection compared with vehicle-injected control fish; pimozide potentiated the effect of LHRH-A between and 12 and 24 h after injection with relatively maximal levels occurring at 24 h after injection (Figure 4). At 20 °C LHRH-A significantly elevated serum concentrations of GtH between 3 and 18 h after injection, compared with vehicle-injected control fish; serum concentrations of GtH in fish injected with a combination of pimozide and LHRH-A were not significantly different from those of fish injected with LHRH-A at respective times after injection (Figure 5). Values are means $\pm$ S.E.M (n=8 to 10). Data were analyzed by Duncan's multiple range test at the $p < 0.05$ level of significance; values sharing a common letter are not significantly different.


12 ° C
Vehicle
Pim
LHRH-A
Pim/LHRH-A
Serum GtH (ng/ ml)
150
100
50
0
3
6
12
18
24
30
48
Time After Injection (h)
G
G
G
EF
DE
DE
E
E
DE
DE
DE
BC
CDE
AB
AB
AB
AB
AB
AB
CD
AB


20 ° C
Vehicle
Pim
LHRH-A
Pim/LHRH-A
Serum GtH (ng/ ml)
125
100
75
50
25
0
0
3
6
12
18
24
Time After Injection (h)
D
CD
CD
CD
CD
CD
CD
C
AB
AB
AB
AB
AB
AB

E. Effect of sampling time on the serum GtH response of goldfish to i.p. injection of domperidone.

Male and female goldfish (sexually regressed, January, 1986) were maintained at 8° C and 8 h photoperiod. At t = 0h fish were segregated into groups , blood sampled, and injected with given doses of domperidone; fish were blood sampled at t = 6h and t = 24h after injection, and the serum was isolated, stored frozen and later analyzed for GtH content, as described in Chapter II. Injection of vehicle had no significant effect on serum concentrations of GtH (data not shown). Domperidone, at all doses tested, significantly increased serum concentrations of GtH, in a dose-related manner, in fish sampled at t = 6h and 24h after injection (Figure 6). Serum concentrations of GtH in fish sampled at t = 6h and 24h after injection were not significantly different. Values are means $\pm$ S.E.M. (n = 7 to 10). Data were analyzed by analysis of variance and Duncan's multiple range test at the $p < 0.05$ level of significance; values with common letters were not significantly different.


B A C D E G F I H J
Serum GtH (ng/ ml)
70
60
50
40
30
20
10
0
A
B
C
D
E
F
G
H
I
J
GtH (t = 6h)
GtH (t = 24h)
0 1 5 10 15 20
Domperidone Dose (nmol/ g BW)

APPENDIX II

Technical aspects of the dopamine radioreceptor assay.

Preliminary studies provided information useful for the design of this radioreceptor assay.

The separation of bound from free radioligand was found to be critical. In initial trials, incubations (1.8 ml vol) were terminated by addition of excess buffer (5 ml) followed by high speed centrifugation (35000 g X 15 min at 4°C). The supernatant was discarded, the pellet briefly rinsed with assay buffer and the pellet recovered from the 13-ml polyallomar tube by rinsing the tube with LSC cocktail. Pellet recovery was inconsistent and displaceable binding was not demonstrable, presumably due to dissociation of bound radioligand during centrifugation. Rapid vacuum filtration was subsequently evaluated as a method of separating bound from free radioligand. Filters were manufactured by "punching-out" 25-mm discs from Whatman GF/B material using a short length of modified high-pressure steampipe (rough stock OD approximately 32.5 mm, ID approximately 23.5 mm) milled to 25 mm ID and having an external bevel at each "cutting end". Filters were contained in Millipore filter holders (Swinnex SX00 025 00; Millipore Corporation, Bedford, MA, U.S.A.) modified assording to Johns and Coons (1983) (Journal of Pharmacological Methods 9, 263-267). Filter holders were mounted on hypodermic needles (25-g, 37 mm), inserted through rubber stoppers (#1; predrilled to accommodate the needle); these filtration units were mounted in a filtration manifold. The filtration manifold, designed to provide equivalent negative pressure to each filtration unit, consisted of a box, constructed of perspex (approximately 11 mm

thick), with approximate dimensions of 12.5 X 50 X 100 cm. The manifold contained internal trusses for support, and had holes milled in the top to accommodate 105 filter holders and a hole for outlet to a vacuum souce via a vacuum trap. Four types of filters (GF/B, GF/C (Whatman), AP-20, AP-40 (Millipore)) were evaluated using the radioreceptor assay protocol described in Chapter III; criteria for evaluation were (i) retention of bound [^{3}H]-spiperone, and (ii) adsorption of ^{3}H-radioactivity to filter material. Of the 4 types tested, AP-20 and GF/B filters consistently retained the greatest amounts of bound [^{3}H]-spiperone and adsorbed the least amount of [^{3}H]-spiperone; GF/B filters were selected on the basis of cost.

Additional methods were evaluated for reducing adsorption of ^{3}H-ligand; filters were pre-soaked in buffer in the absence or presence of 0.2 % bovine serum albumin (BSA), 0.2 % BSA was used in the assay buffer, and filters were siliconized (Siliclad, Fisher Scientific). The presence of BSA at any stage of the assay increased adsorption and depressed apparent specific [^{3}H]-spiperone binding by over 50 %; siliconizing filters had a similar effect. In contrast, presoaking filters for 36 h in assay buffer without BSA increased retention of bound [^{3}H]-spiperone, decreased non-specific binding and decreased variation in binding between replicates.

Rinsing filters with assay buffer is commonly used to remove free radioligand. Repeated rinsing with 5-ml aliquots of assay buffer had little effect on adsorbed or non-specifically bound [^{3}H]-spiperone (bound in the presence of excess domperidone) but progressively reduced the amount of total bound [^{3}H]-spiperone (bound in the absence of competitor). After rinsing, filters were routinely dried in LSC vials in

an airstream at room temperature to remove moisture that would otherwise cloud the toluene-based LSC-cocktail. [^{3}H]-Spiperone was not lost during drying. Equivalent aliquots of [^{3}H]-spiperone in ethanol were applied to buffer soaked-filters in scintillation vials, and to another set of filters which had been were dried prior to receiving cocktail; there was no significant variation in the amount of ^{3}H-radioactivity or quenching between the 2 sets of vials.

Several goldfish pituitary tissue preparations were evaluated for use in this radioreceptor assay. Comparisons were made with (i) whole pituitary homogenate, (ii) post-centrifugation (27000 g X 20 min at 4^{o}C) supernatant, and (iii) 27000 g resuspended pellet; the criteria for evaluation were the magnitude of total and specific binding and domperidone displacement of [^{3}H]-spiperone bound to each tissue preparation. The ratio of total:non-specifically bound [^{3}H]-spiperone was similar among the tissue preparations examined; the supernatant and pellet had less [^{3}H]-spiperone bound than homogenate so that 2-pituitary equivalents/tube of pellet preparation were required to accumulate a significant number of counts. Also, the profile of domperidone displacement of [^{3}H]-spiperone was similar among the tissue preparations tested (data from subsequent trials). Whole pituitary homogenate was routinely used in assays; homogenate from frozen then thawed pituitaries was not used as freezing/thawing reduced specific binding.

The effect of temperature on the time course of total and specific binding was studied. Whole pituitary homogenate was incubated with [^{3}H]-spiperone at 4, 12, 20 or 37 ^{o}C; incubation was terminated at approximately 0, 20 45, 90, 135, 274 min or 24 h. At 4 ^{o}C, total and specific binding increased with time to acheive relatively maximal

levels, approximately 10 and 5 % of total radioactivity, respectively, between 1 and 2 h incubation; binding decreased gradually thereafter. At higher incubation temperatures the rate at which binding reached maximum equilibrium levels was increased; for example, maximum binding at 12 °C occurred at approximately 20 min incubation. Increased temperature decreased the magnitude of binding and increased the loss of bound ^{3}H-radioactivity after maximal levels were achieved; for example, at 12 °C maximal total and specific binding were 8 and 3 % of total radioactivity, whereas at 20 and 37 °C binding was reduced to almost undetectable levels. Prolonged incubation (greater than 1 h) at 4 °C depressed specific binding.